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14. **ABSTRACT**
Since the initiation of this proposal, we have demonstrated that while LPP1 and LPP3 mRNA levels are decreased approximately 2 fold in breast cancer cells, autotaxin levels are increased approximately 28 fold in breast cancer cells isolated directly from patients. This should result in increased LPA and S1P production by breast cancer cells in patients. Using a novel enzyme activity assay, we have demonstrated that autotaxin acitivity is not significantly different in blood from control and breast cancer patients. Thus, the increased mRNA levels in tumor cells are not translated into increased autotaxin activity in the blood stream. We have demonstrated that down regulation of autotaxin by RNAi results in a decreased signaling, a novel S phase arrest and apoptosis in breast cancer cells .We have utilized a novel Si1P antibody to neutralize S1P in vitro and are currently treating mice with breast cancer xenografts to determine effects on cell growth. We have established transgenic mice expression the three LPA receptors as well asautotaxin in breast epithelium. We have obtained a LPP transgenic mouse to determine the effects of degradation of LPA and S1P on breast function and tumorigenesis by crossing to the above mice and to tumor prone mice.

15. SUBJECT TERMS
Autotaxin, lysophosphatidic acid, sphingosine 1 phosphate, lysoPLD,

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Introduction

This application was originally for fellowship support of Makiko Umezu-Goto. Dr. Goto unexpectedly had to return to Japan in December 2004. Her return was based on a family need. With the support and productivity of this fellowship, Makiko now has a faculty position in Japan. Following the great success of this support in career development for this outstanding fellow, we petitioned for the transfer of support to Shuying Liu, who is taking over the studies of this proposal. Dr. Goto developed the constructs and organized injection of embryos and transplantation into the mice. She also began the process of identifying which transgenic mice expressed the construct. Dr. Shuying Liu has continued the project since Makiko's departure in December 2004. This report formalizes the request for transfer of this fellowship to Dr. Liu.

Background

Multiple different bioactive lysophospholipids, including lysophosphatidic acid (LPA), lysophosphatidylcholine (LPC), sphingosylphosphorylcholine (SPC), sphingosine 1 phosphate (S1P) and lysophosphatidylserine (LPS) exhibit pleiomorphic effects on multiple cell lineages including breast cancer cells. LPA and S1P signal cells through specific cell surface receptors of the EDG family of cell surface G protein coupled receptors (GPCR), whereas SPC and LPC activate the OGR1 family of GPCR. Further LPC, LPS and LPA activate breast cancer cells as indicated by increases in tyrosine phosphorylation, cytosolic calcium, and phosphorylation of the p70S6, ERK and JNK kinases. The effects of LPA and LPC on intracellular signaling in breast cancer cells is translated in to functional changes such as increases in production of multiple growth factors from breast cancer cells including interleukin 6 and 8, which are potent regulators of neovascularization and activation of the AP-1 transcription complex. Goetzl and colleagues have demonstrated that lysophospholipids can increase the proliferation of breast cancer cells. In support of a role for lysophospholipids in signaling in breast cancer, multiple EDG receptors are aberrantly expressed in breast cancer cells.

The mechanisms regulating the production and degradation of lysophospholipids are just beginning to be elucidated. The most likely pathway for LPA production is the conversion of membrane phosphatidylcholine (PC) to LPC by the action of PLA1 or PLA2. LPC is converted to LPA by lysophospholipase D (lysoPLD) aka Autotaxin. Autotaxin is a major regulator of cellular motility and invasion. Further high levels of autotaxin correlate with aggressiveness and metastatic capacity of breast cancer cell lines. We have demonstrated that lysoPLD converts LPC to LPA and the resultant LPA induces cellular proliferation, cellular survival and cellular motility and chemotaxis. LPA, in turn, is degraded by lysophosphatidic acid phosphatases (LPP) to monoacylglycerol.

Objective/Hypothesis

An improved understanding of the production, metabolism and function of lysophosphatidic acid (LPA) and sphingosine 1 phosphate (S1P) in breast cancer could lead to the identification of novel markers or targets for therapy.

Specific Aims

(1) To determine the mechanisms regulating the production and metabolism of LPA and S1P in breast cancer

(2) To determine the interplay between LPA and S1P in the proliferation, survival, invasion and metastases of breast cancer

(3) To determine whether the production or action of LPA and S1P are targets for therapy in breast cancer

Relevance

Lysophospholipids appear to play an important role in the initiation and progression of breast cancer. The enzymes producing these lysophospholipids as well as their receptors are targets for therapy in breast cancer.

Body

We have made significant progress on each of the aims in this proposal.

We have determined lysophospholipid phosphohydrolases (LPP1, 2, 3) and autotaxin (ATX) levels in breast cancer cell lines with QPCR and in breast cancer patients by transcriptional profiling (1). While LPP1 and LPP3 is decreased approximately 2 fold in tumor cells, ATX is increased approximately 28 fold in breast cancer cells directly from patients (1). This should result in increased LPA and S1P production by breast cancer cells in vivo.

Using a novel enzyme activity assay, we have demonstrated that autotaxin activity is not significantly different between sera and plasma from control and breast cancer patients (1). Thus, the increased mRNA levels in tumor cells are not translated into increased autotaxin activity in the blood stream.

We have demonstrated that downregulation of autotaxin by RNAi results in a decreased signaling, a novel S phase arrest and apoptosis in breast cancer cells (Manuscript in preparation). We are currently determining the effects of over and underexpression of LPP1s in proliferation and survival of breast cancer cells in vitro. We will establish stable breast cancer cell lines with over or underexpression of ATX or LPPs and determine the effects on growth in vivo. If constitutive stable cell lines cannot be developed, we will develop conditionally expressing cell lines. We have developed a LPP expressing adenovirus and will determine the effects of "gene therapy" with this virus on in vivo tumor growth. (Studies in progress by Shuying Liu).

We have obtained a S1P neutralizing antibody. We have used this antibody to neutralize S1P in vitro and are currently treating mice with breast cancer xenografts to determine effects on cell growth (Studies in progress by Shuying Lu).

We have obtained a series of agonists and antagonists of LPA receptors. With these agonists and antagonists, we have demonstrated that growth, motility and production of neovascularization factors including IL8, IL6 and VEGF is mediated by specific LPA receptors in breast cancer cells (Studies in progress by Shuying Liu).

We have established transgenic mice expression the three LPA receptors as well as autotaxin in breast epithelium. We have obtained a LPP transgenic mouse to determine the effects of degradation of LPA and S1P on breast function and tumorigenesis by crossing to the above mice and to tumor prone mice (Studies in progress by Shuying Lu).

Key Research Accomplishments

1. LPP1 and LPP3 mRNA levels are decreased approximately 2 fold in breast cancer cells directly from the patient
2. Autotaxin mRNA is increased approximately 28 fold in breast cancer cells directly from patients (1).
3. Autotaxin activity is not significantly different between sera and plasma from control and breast cancer patients (1).
4. Downregulation of autotaxin by RNAi results in a decreased signaling, a novel S phase arrest and apoptosis in breast cancer cells (Manuscript in preparation).
5. We have demonstrated that neutralizing S1P in vitro decreases growth of some but not all breast cancer cell lines (Studies in progress by Shuying Lu).
6. We have established transgenic mice expression the three LPA receptors as well as autotaxin in breast epithelium. (Studies in progress by Shuying Lu
7. We have obtained a LPP transgenic mouse (Studies in progress by Shuying Lu).

Reportable outcomes

Umezu-Goto, M., Tanyi, J., Lahad, J., Liu, S., Yu, S., Lapushin, R., Hasegawa, Y., Lu, Y., Trost, R., Bevers, T., Jonasch, E., Aldape, K., Liu, J., James, R.A., Ferguson, C.G., Xu, Y., Prestwich, G.D., and Mills G.B., 2004 Lysophosphatidic acid production and action: Validated targets in cancer?. J. Cellular Biochemistry 92:1115-40.

The following manuscript was facilitated by the support from this fellowship but is not a direct reportable outcome.

Hasegawa, Y., Umezu-Goto, M. and Mills GB 2004 Lysophosphatidic acid (LPA) analogs, D-3-deoxy-phosphophatidyl-*myo*-inositol ether lipid (DPIEL) and lysophosphatidylglycerol (LPG), antagonize LPA receptor activation Submitted.

Conclusions

The overarching goal of the training support was to train exciting new investigators in breast cancer research through validating S1P and LPA production and action as potential targets for therapy in breast cancer.

References

1. Umezu-Goto, M., Tanyi, J., Lahad, J., Liu, S., Yu, S., Lapushin, R., Hasegawa, Y., Lu, Y., Trost, R., Bevers, T., Jonasch, E., Aldape, K., Liu, J., James, R.A., Ferguson, C.G., Xu, Y., Prestwich, G.D., and Mills G.B., 2004 Lysophosphatidic acid production and action: Validated targets in cancer?. J. Cellular Biochemistry 92:1115-40.

Appendices

1. Umezu-Goto, M., Tanyi, J., Lahad, J., Liu, S., Yu, S., Lapushin, R., Hasegawa, Y., Lu, Y., Trost, R., Bevers, T., Jonasch, E., Aldape, K., Liu, J., James, R.A., Ferguson, C.G., Xu, Y., Prestwich, G.D., and Mills G.B., 2004 Lysophosphatidic acid production and action: Validated targets in cancer?. J. Cellular Biochemistry 92:1115-40.

Lysophosphatidic Acid Production and Action: Validated Targets in Cancer?

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Abstract The completion of the human genome project, the evolution of transcriptional profiling and the emergence of proteomics have focused attention on these areas in the pathophysiology and therapy of cancer. The role of lysophospholipids as potential mediators in cancer pathophysiology, screening and management has taken a major leap forward with the recent cloning of several enzymes involved in the metabolism of lysophospholipids. Lysophospholipids, although small molecules, contain a high "informational" content. Differences include the nature of the phosphate head group, the regiochemistry of the fatty acyl chain on the glyceryl backbone, the presence of ether versus ester linkages to the backbone, and the length and saturation of the fatty acyl or alkyl chain. This informational content is sufficient to result in a marked structure function activity relationship at their cognate receptors. Thus the emerging discipline of "functional lipidomics" is likely to prove as important as genomics and proteomics in terms of early diagnosis, prognosis, and therapy. Lysophospholipid levels are elevated in vivo in a number of pathophysiological states including ascitic fluid from ovarian cancer patients indicating a role in the pathophysiology of this devastating disease. Although controversial, levels of specific lysophospholipids may be altered in the blood of cancer patients providing a potential mechanism for early diagnosis. Several of the enzymes involved in the metabolism of lysophospholipids are aberrant in ovarian and other cancers. Further, the enzymes are active in the interstitial space, rendering them readily accessible to the effects of inhibitors including antibodies, proteins, and small molecules. In support of a role for lysophospholipids in the pathophysiology of cancer, expression of receptors for lysophospholipids is also aberrant in cancer cells from multiple different lineages. All of the cell surface receptors for lysophospholipids belong to the G protein coupled receptor family. As over 40% of all drugs in current use target this family of receptors, lysophospholipid receptors are highly "druggable." Indeed, a number of highly specific agonists and antagonists of lysophospholipid receptors have been identified. A number are in preclinical evaluation as therapeutics. We look forward to the next several years when the role of lysophospholipids in physiology and the pathophysiology and management of cancer and other diseases are fully elucidated. J. Cell. Biochem. 92: 1115–1140, 2004. © 2004 Wiley-Liss, Inc.

Key words: lysophosphatidic acid; autotaxin; cancer; treatment

Overview

Lysophosphatidic acid, the simplest phospholipid, exhibits a broad spectrum of activity in many different cellular lineages and contributes to multiple physiological and likely to many pathophysiological processes in vivo [see Fang et al., 2002; Graler and Goetzl, 2002; Lynch and Macdonald, 2002; Mills et al., 2002; Yang et al., 2002a; Feng et al., 2003; Luquain et al., 2003a; Mills and Moolenaar, 2003; Tigyi and Parrill, 2003; Xu et al., 2003 for reviews and

elsewhere in this issue]. LPA mediates it effects by binding to G protein coupled receptors with subsequent activation of heterotrimeric G proteins and downstream events including increases in cytosolic calcium, alterations in cAMP levels, Rac and Rho small GTPases, activation of phospholipase C, protein kinase C, the phosphatidylinositol 3 kinase pathway, the RAS MAPK pathway, proteases and, by as yet somewhat unclear mechanisms, tyrosine kinases [Fang et al., 2002; Graler and Goetzl, 2002; Lynch and Macdonald, 2002; Mills et al., 2002; Yang et al., 2002a; Feng et al., 2003; Luquain et al., 2003a; Mills and Moolenaar, 2003; Tigyi and Parrill, 2003; Xu et al., 2003]. This latter effect is likely due, in part, to the activation of proteases and release of cell surface growth factors with subsequent activation of the epidermal growth factor receptor family [Prenzel et al., 1999; Gschwind et al., 2003]. There are four different LPA receptors so far characterized on the surface of mammalian cells, three members of the Edg family of receptors (LPA1, 2, 3 previously known as Edg 2, 4, 7, respectively), and a newly identified receptor LPA4 (previously GPR23/P2Y9) of the purinergic receptor family [Fang et al., 2002; Graler and Goetzl, 2002; Lynch and Macdonald, 2002; Mills et al., 2002; Yang et al., 2002a; Feng et al., 2003; Luquain et al., 2003a; Mills and Moolenaar, 2003; Noguchi et al., 2003; Tigyi and Parrill, 2003; Xu et al., 2003]. Through activation of these GPCR and subsequent downstream signaling, LPA induces cellular proliferation, cellular differentiation, regulates cell–cell interactions, inhibits cell death, increases cellular motility, increases invasiveness, increases the production of cytokines including those affecting formation and maintenance of new vessels, and increases the production and action of proteases [Fang et al., 2002; Graler and Goetzl, 2002; Lynch and Macdonald, 2002; Mills et al., 2002; Yang et al., 2002b; Feng et al., 2003; Luquain et al., 2003a; Mills and Moolenaar, 2003; Tigyi and Parrill, 2003; Xu et al., 2003]. On an organism level, LPA is implicated in complex physiological states such as immunological competence, brain development, wound healing, coagulation, and regulation of blood pressure [Fang et al., 2002; Graler and Goetzl, 2002; Lynch and Macdonald, 2002; Mills et al., 2002; Yang et al., 2002a; Feng et al., 2003; Luquain et al., 2003a; Mills and Moolenaar, 2003; Tigyi and Parrill, 2003; Xu

et al., 2003]. LPA has recently been implicated as a physiological ligand for PPARγ, a member of the nuclear hormone receptor superfamily [McIntyre et al., 2002]. Recently, LPA was shown to induce neointima formation—a prelude to atherosclerosis in humans—by interaction with PPARγ in a rat carotid artery model [Zhang et al., 2004]. Importantly, LPA analogues that were inactive at the GPCRs were potent activators of PPARγ in this assay [Xu and Prestwich, 2002], demonstrating that LPA acting on this nuclear transcription factor was both necessary and sufficient for neointima formation.

As the effects of LPA are pleiomorphic and could potentially contribute to the development of multiple pathophysiological states, under normal circumstances the production and degradation of LPA is in a tight equilibrium with normal levels of LPA being in the 100–200 nM range or lower [Xu et al., 1998; Xiao et al., 2000; Shen et al., 2001; Aoki et al., 2002; Baker et al., 2002; Sano et al., 2002]. Levels of LPA are elevated during wound healing and in a number of physiological media such as saliva and ovarian cyst fluid [Westermann et al., 1998; Sugiura et al., 2002]. The physiological functions of LPA suggest that LPA could contribute to a number of pathophysiological states including cancer, autoimmune or immunodeficiency disease, atherosclerosis, and ischemia reperfusion injury [Fang et al., 2002; Graler and Goetzl, 2002; Mills et al., 2002; Yang et al., 2002a; Feng et al., 2003; Luquain et al., 2003a; Mills and Moolenaar, 2003; Okusa et al., 2003; Tigyi and Parrill, 2003; Xu et al., 2003]. Indeed as described above, the physiological responses to LPA parallel events, which must occur for the full expression of the malignant phenotype including the ability to invade and metastasize [Hanahan and Weinberg, 2000; Fidler, 2003]. Although the underlying mechanisms remain elusive, aberrations in production and degradation of LPA have been identified in cancer cells as well as in cancer patients, in particular in the markedly elevated levels of LPA in the ascitic fluid of ovarian cancer patients and in autocrine activation loops in ovarian and prostate cancer [Shen et al., 1998; Eder et al., 2000; Fang et al., 2002; Mills et al., 2002; Xie et al., 2002; Feng et al., 2003; Mills and Moolenaar, 2003; Sengupta et al., 2003]. In combination with alterations in LPA receptor expression and potentially receptor function in multiple cancer

lineages, this implicates LPA as an important mediator in the pathophysiology of cancer [Goetzl et al., 1999; Fang et al., 2002; Mills et al., 2002; Feng et al., 2003; Mills and Moolenaar, 2003]. As a corollary, LPA production and degradation, receptors and signaling are high quality targets for therapy [Feng et al., 2003; Mills and Moolenaar, 2003].

LPA Production

LPA is a critical component of the production and remodeling of lipids that occurs intracellularly. Both calcium-independent phospholipase A2 (iPLA2) and calcium-dependent PLA2 (cPLA2) contribute to this process intracellularly (Fig. 1). Intracellular LPA can activate the PPARγ receptor, however, the physiologic role of this process remains to be clarified [McIntyre et al., 2002; Zhang et al., 2004]. Through conversion to PA, LPA also regulates membrane curvature and formation of caveoli, which are critical to internalization and sorting of signaling complexes [Schmidt et al., 1999; Kooijman et al., 2003]. The unconventional lipid lysobisphosphatidic acid (LBPA) has recently been shown to induce the formation of multi-vesicular liposomes in vitro, and, with a protein partner Alix, the organization of endosomes in vivo [Matsuo et al., 2004]. Thus, by activating intracellular receptors and by serving as a substrate for enzymes such as endophilin and LCAT, LPA plays an important role in cellular functions (Fig. 1). Whether, under physiological conditions, extracellular LPA migrates to the cytosol in sufficient concentrations to contribute to these processes or whether intracellularly produced LPA is the only relevant source remains to be determined. Further, it is not clear that intracellular LPA is exported from the cell to participate in intercellular signaling. Indeed a series of secreted and ectoenzymes with their catalytic surface outside the cell appear critical to the production and metabolism of extracellular LPA.

As noted above, under physiological conditions, plasma LPA levels are maintained at low concentrations in the range of 100–200 nM [Xu et al., 1998; Xiao et al., 2000; Shen et al., 2001; Aoki et al., 2002; Baker et al., 2002; Sano et al., 2002]. LPA levels in plasma represent the steady state attained by separately regulated rates of production, degradation, and clearance. The removal of LPA from the bloodstream is extremely rapid, suggesting that degradation or clearance is very efficient. In addition to containing low levels of LPA, inhibitory factors in plasma limit LPA activity and production [Sano et al., 2002]. Thus the bioavailable levels of LPA in plasma may be below those able to optimally activate LPA receptors. Nevertheless, plasma contains both the enzymes and the substrates required for LPA production [Aoki et al., 2002; Sano et al., 2002]. Incubation of plasma at 37°C, e.g., results in an increase in LPC levels and a concomitant LPA increase resulting in the production of micromolar amounts of LPA [Aoki et al., 2002].

Cellular activation such as occurs during blood clotting, wound healing, or inflammation increases local production and action of LPA. Serum, in comparison to plasma, contains much higher levels of LPA with levels being between 1 and 5 μm [Aoki et al., 2002; Sano et al., 2002]. In tissue culture, it is likely that continuous production of LPC and LPA results in regeneration of LPA over time. Indeed, the regeneration of LPA, a potent growth and survival factor likely accounts for the quality of serum as a growth media. Platelets were initially proposed to be the main source of LPA in serum as they can produce LPA on activation [Gerrard and Robinson, 1989]. However, the amount of LPA released by activated platelets is insufficient to explain the increase in LPA levels that occur on blood clotting and likely accounts for only a small portion of the LPA present in serum [Aoki et al., 2002; Sano et al., 2002]. Further the fatty acyl chain composition of LPA produced by platelets [Gerrard and Robinson, 1989] $16:0 > 18:0 > 20:4 > 18:1 > 18:2$ is markedly different from that present in plasma ($18:2 > 18:1 = 18:0 > 16:0 > 20:4$) or serum ($20:4 > 18:2 > 16:0 = 18:1 > 18:0$) [Sano et al., 2002]. This suggests that different molecular processes regulate production or degradation of LPA by platelets and the production and degradation of LPA that occurs in plasma or during the coagulation process that produces serum. LPA1 and -2 demonstrate selectivity towards saturated LPA as ligands, whereas LPA3 and -4 preferentially bind unsaturated versions of LPA [Bandoh et al., 1999; Yang et al., 2002a; Noguchi et al., 2003; Tigyi and Parrill, 2003]. Thus the different forms of LPA produced by platelets and during coagulation could activate different species of LPA receptors resulting in differential functions.
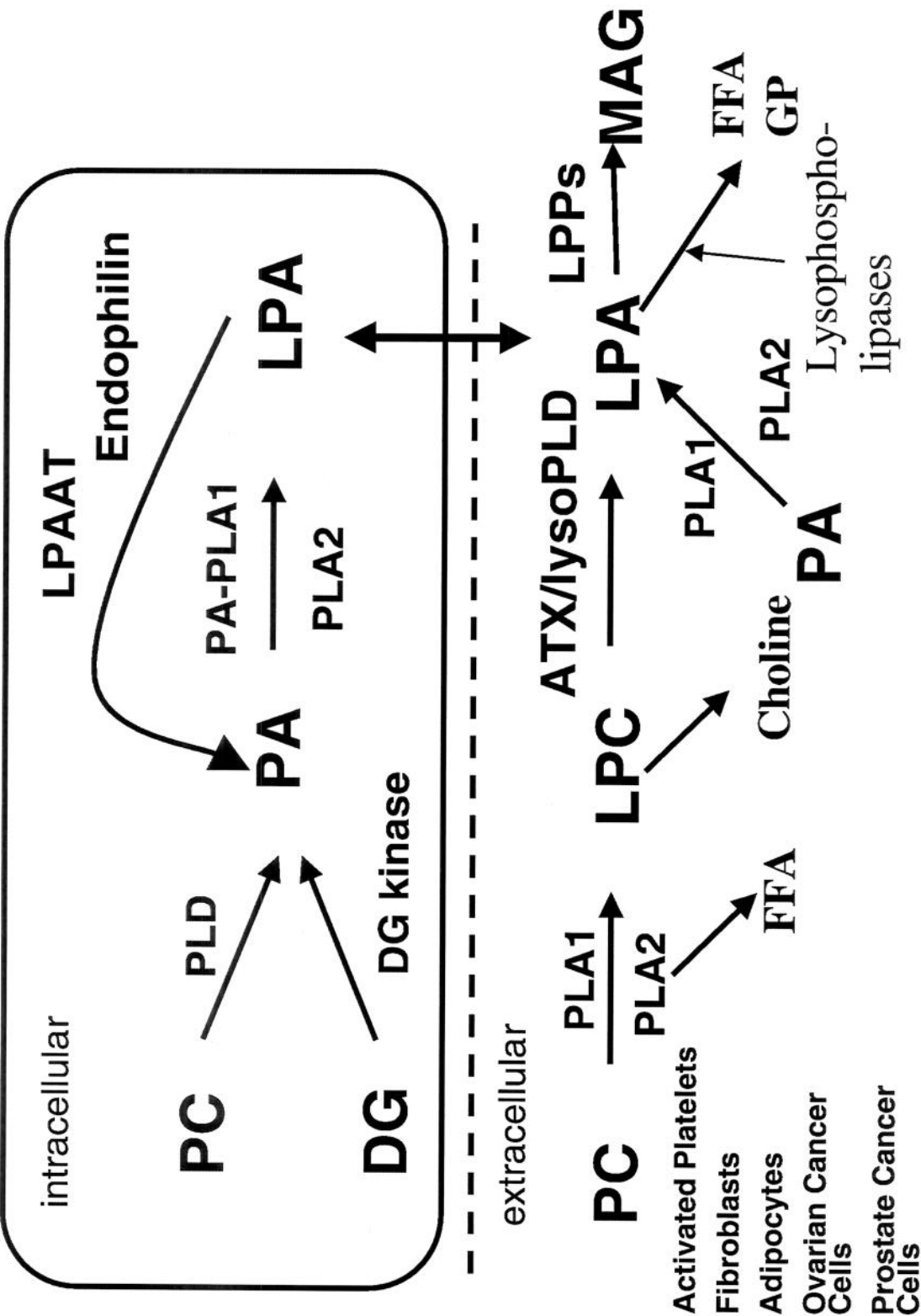
Fig. 1. LPA metabolism pathways the major intracellular and extracellular pathways of production and degradation of LPA are outlined. Whether intracellular and extracellular LPA are independent pools or whether there is exchange between these pools is unknown. Intracellular and extracellular LPA mediate different but important functions that could contribute to the pathophysiology of cancer. Cells known to produce LPA are indicated at the lower left.

Platelets contain high levels of phospholipases capable of cleaving phospholipids to produce lysophospholipids [Aoki et al., 2002; Sano et al., 2002]. Group IIA secretory phospholipase A2 (sPLA2, pancreatic) and phosphatidylserine-specific phospholipase A1 (PS-PLA1) are released from platelets activated by thrombin or calcium ionophores and likely contribute to the production of LPA by producing lysophosphatidylcholine (LPC), lysophosphatidylethanolamine (LPE), lysophosphatidylinositol (LPI), and lysophosphatidylserine (LPS) precursor molecules [Aoki et al., 2002; Sano et al., 2002]. PS-PLA1 produces sn-2 lysophospholipids with unsaturated fatty acyl chains. At either high or low pH, the sn-2 fatty acyl chain migrates to the sn-1 site with equilibrium favoring sn-1 lysophospholipids, however, at neutral pH sn-2 lysophospholipids are relatively stable. As noted above, these forms of LPA may selectively activate LPA3 and -4 [Bandoh et al., 1999; Noguchi et al., 2003]. In addition to sPLA2 IIA, it is also likely that several other isoforms of sPLA2 can contribute to LPA production following platelet activation or in serum as expression of sPLA2 isoforms in platelets is species dependent and also LPA production is not altered in mice genetically deficient in group IIA sPLA2 [Fourcade et al., 1998].

sPLA2 has limited ability to hydrolyze lipids in intact cell membranes [Kudo et al., 1993; Fourcade et al., 1995, 1998]. sPLA2 selectively hydrolyzes lipids present in damaged membranes, membranes of activated cells or microvesicles such as those released during apoptosis or produced by cancer cells to produce sn-1 lysophospholipids [Kudo et al., 1993; Fourcade et al., 1995, 1998]. Disruption of the membrane microenvironment by phosphatidic acid (PA) produced by the action of phospholipase (PLD) or activation of intracellular signaling pathways such as thrombin-induced increases in cytosolic calcium render cellular membranes more susceptible to the action of sPLA2 [Kudo et al., 1993; Fourcade et al., 1995, 1998; Kinkaid et al., 1998]. PLD also induces the production of extracellular microvesicles that are susceptible to the effects of sPLA2 [Morgan et al., 1997]. Strikingly, LPA induces the activation of PLD, increases cytosolic free calcium and increases PA levels compatible with LPA contributing to its own production [van der Bend et al., 1992; Kam and Exton, 2004]. The microenvironment

wherein PS-PLA1 can hydrolyze lipids is not clear but it may also require access to substrates in particular structures limiting the production of LPA in plasma. Taken together, this suggests that LPA production likely occurs at sites of cellular injury or disruption. Further as tumors exhibit high levels of spontaneous apoptosis that produces vesicles, production of cellular vesicles and aberrations in membrane composition and symmetry [Fourcade et al., 1995; Ginestra et al., 1999; Andre, 2002], LPA may be produced at elevated levels in the tumor microenvironment.

In comparison to LPA, plasma contains high levels of phospholipids and lysophospholipids [LPC levels exceeding 100 μM and phosphatidylcholine (PC) levels approaching 1 mM]. Thus, precursors for LPA production are abundant. Indeed, incubation of plasma or serum at 37°C results in a time dependent increase in both LPC and LPA [Aoki et al., 2002]. However, despite the high concentrations of precursors, LPA levels are maintained at a low level. LPA production by plasma may be constrained in an inactive state by the presences of inhibitors [Sano et al., 2002]. This is consistent with a decreased rate of production of LPA in plasma as compared to serum [Aoki et al., 2002].

The source of phospholipids and lysophospholipids in plasma and sera that contribute to LPA production under physiological and pathophysiological conditions is not completely clear. Hepatocytes produce large amounts of lipids and lysophospholipids. LDL and HDL contain phospholipids, which can be converted to lysophospholipids by oxidation or by the action of sPLA2 or PS-PLA1 [Natarajan et al., 1995; Siess, 1999]. Indeed a number of responses to oxidized LDL or lysophospholipids such as LPC could potentially be mediated by conversion to LPA [Natarajan et al., 1995; Siess, 1999]. The still controversial suggestion that LPC-specific GPCRs exist [Kabarowski et al., 2001; Zhu et al., 2001; Bektas et al., 2003; Ludwig et al., 2003] suggests that LPC may mediate cellular signaling in addition to being a precursor for LPA.

A number of cell types produce lysophospholipids under physiological conditions. In blood cells, activation of platelets with thrombin or calcium ionophores results in the release of lysophospholipids [Aoki et al., 2002; Sano et al., 2002]. Erythrocytes can release small amounts of LPC. Both lecithin cholesterol acyltransferase

and platelet-activating factor acetylhydrolase (PAF-AH) have been implicated in production of LPC in plasma and sera. Studies by Aoki and colleagues using blood from patients deficient in LCAT and PAF-AH demonstrate that both LPC and LPA production is deficient in plasma from LCAT-deficient but not PAF-AH deficient patients [Aoki et al., 2002]. However, it is important to note that while basal LPC levels are slightly depressed in LCAT-deficient patients, they still are in the 100 μM range suggesting that plasma LPC originates from a source other than LCAT. Further the 100 μM of LPC present in plasma is far in excess of that required for the production of LPA. Patients with LCAT deficiency exhibit kidney dysfunction, anemia, and corneal opacification along with aberrations in plasma composition and amounts of cholesterol and other lipids [Gjone, 1982]. It is not clear whether LCAT-deficient patients exhibit decreased agonist induced LPA production in pathophysiological states and whether any of the consequences of the syndrome are due to LPA-deficiency. Indeed, these patients do not exhibit aberrations in processes proposed to be mediated by LPA such as wound healing or vessel development [Gjone, 1982].

In an independent pathway, PA produced by the action of PLD on cellular membranes can be hydrolyzed by sPLA2 or PA-specific PLA1 (PA-PLA1) to produce LPA [Hiramatsu et al., 2003]. The magnitude of the contribution of this pathway in physiologic or pathophysiologic conditions remains unknown (Fig. 1). A fluorogenic PLA1/2 assay using a PC analogue has been developed [Feng et al., 2002], and a fluorogenic substrate for PA-specific PLA activity has also been synthesized [Xu et al., 2004]. These assays can be used to determine activity or screen for inhibitors.
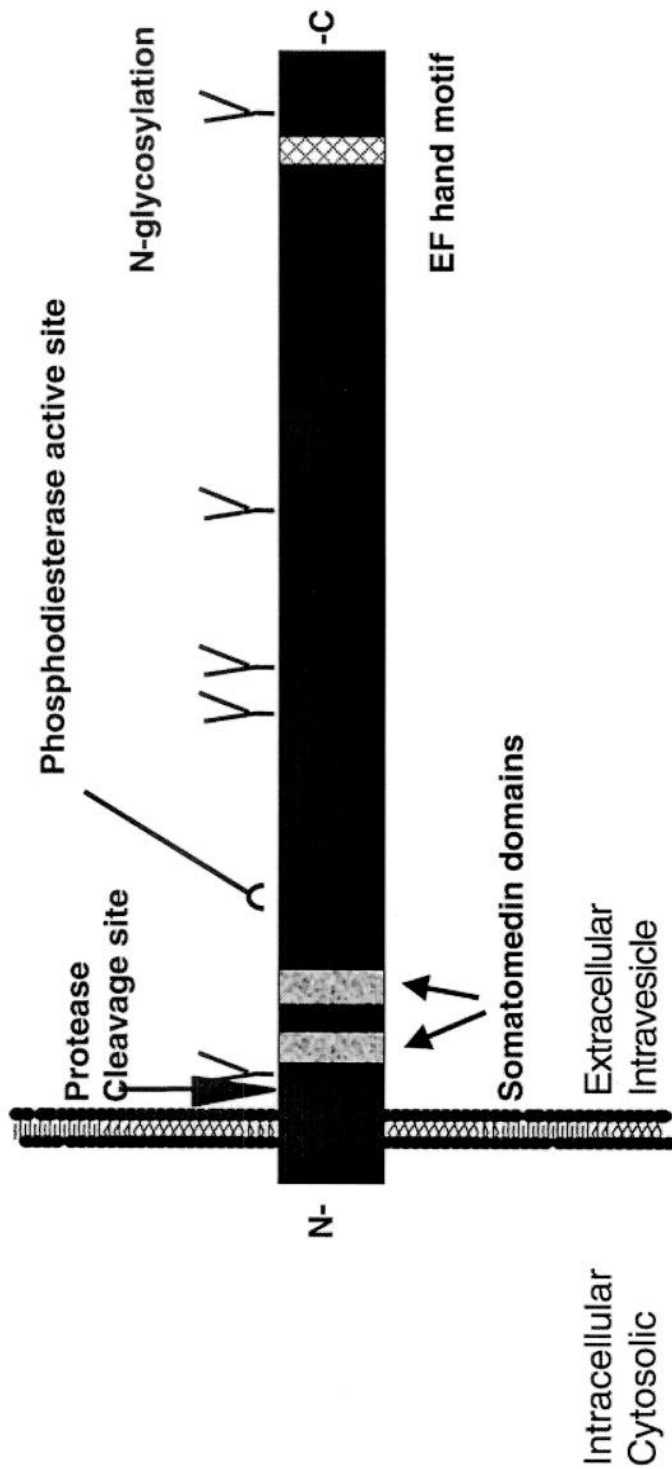
LysoPLD/Autotaxin

Plasma and sera contain a lysophospholipid specific PLD (lysoPLD), which cleaves lysophospholipids to produce LPA [Xu et al., 1998; Aoki et al., 2002]. LysoPLD exhibits a preference for lysophospholipids containing unsaturated fatty acyl chains and may thus contribute to the preferential increases in the unsaturated fatty acyl chain containing LPA present in plasma and sera. LysoPLD activity is increased in women in the third trimester of pregnancy and in women at risk for preterm labor suggesting that levels of lysoPLD can vary under

physiological and pathophysiological conditions [Tokumura et al., 2002a]. LysoPLD activity is also increased in rabbits fed a high cholesterol diet [Tokumura et al., 2002b]. As LPA also increases attachment of monocytes to vascular endothelial cells, and alters proliferation, and differentiation, of vascular smooth muscle cells and is present in atherosclerotic plaques, lysoPLD with subsequent LPA production may contribute to atherosclerosis [Tokumura et al., 1994; Siess et al., 1999].

The cloning of LysoPLD and its identification as autotaxin (ATX, nucleotide phosphodiesterase/pyrophosphatase, NPP2, phosphodiesterase-Ialpha) by the Aoki and Tokumura groups [Tokumura et al., 2002c; Umezu-Goto et al., 2002] opened a new era in our understanding of the metabolism and, significantly, the function of LPA. ATX had been previously cloned from melanoma cells and studied by the Liotta group and others as autocrine motility factor, an inducer of cell motility, angiogenesis, invasiveness, metastasis, and tumor aggressiveness [Murata et al., 1994; Nam et al., 2000, 2001; Yang et al., 2002b]. Indeed, in retrospect, the actions and mechanisms of signaling of ATX and LPA demonstrated remarkable concordance suggesting an overlap in mechanisms. ATX is a member of the nucleotide phosphodiesterase/pyrophosphatase family, but in contrast to other members of the family, it exhibits the novel ability to hydrolyze LPC and sphingosylphosphorylcholine (SPC) to produce LPA and sphingosine 1-phosphate (S1P). All of the demonstrated activities of ATX appear to be related to its ability to hydrolyze lysophospholipids rather than to its nucleotide phosphodiesterase/pyrophosphatase activity [Koh et al., 2003]. At least in model systems, the relative production of S1P by ATX can inhibit the migratory effects of LPA suggesting that the outcome of autotaxin action may reflect the relative production of LPA and S1P [Clair et al., 2003].

ATX, a 125 kDa type II transmembrane protein, has a short intracellular domain and a large extracellular domain with the catalytic site localized near the membrane in the extracellular domain and thus readily accessible to extracellular LPA and S1P as well as to inhibitors and drugs (Fig. 2). While the mechanism of catalysis of ATX is well characterized [Koh et al., 2003], its post-translational processing, regulation of expression and mechanism of

N-glycosylation

Phosphodiesterase active site

EF hand motif

Protease
Cleavage site

Somatomedin domains

Extracellular
Intravesicle

Intracellular
Cytosolic

N-

-C

- **125 kDa type II membrane protein and released from membrane.**
- **5'-nucleotide pyrophosphatase / phosphodiesterase family (NPP-2)**
- **Widely expressed**
- **Upregulated by bFGF, BMP-2, retinoic acid and Wnt-1signaling**
- **Stimulates tumor cell motility, metastasis and angiogenesis**
- **Metastatic capability relates to ATX levels in breast cancer**
- **Ectoenzyme Potential Potential therapeutic target**

Fig. 2. Characteristics of ATX/LysoPLD ATX is released from cells due to perimembrane cleavage. The mechanism and whether this happens intracellularly or at the cell surface is unknown as is the enzyme involved. The major action site of action of ATX is nevertheless extracellular.

release from cells are not well understood. Further, the relative activity of cell surface versus cleaved ATX is unknown. It is also not clear whether cleavage of ATX occurs in intracellular organelles or at the cell surface. Histochemical analysis suggests that that majority of ATX is localized in intracellular organelles rather than on the cell surface (not presented). However, as noted above, ATX and lysoPLD activity is found in bodily fluids and cell supernatants indicating that autotaxin is released from cells either following cleavage in organelles.

As assessed by SAGE analysis (http://cgap. nci.nih.gov/Sage/Viewer), levels of ATX are low in most cell lineages. Spinal cord has the highest level of expression of ATX suggesting a novel function in differentiated cells. Significant levels of ATX mRNA are also present in brain, breast, prostate, and hematopoietic cells. ATX can be upregulated by a number of cellular stimuli including growth factors such as bFGF and BMP2, retinoic acid and the WNT pathway [Bachner et al., 1998; Tice et al., 2002], however, the role of ATX and LPA in the function of these mediators is unknown. ATX can regulate proliferation of preadipocytes, and its production is increased during adipocyte differentiation and obesity implicating LPA production in obesity [Gesta et al., 2002; Ferry et al., 2003]. The mechanisms regulating ATX upregulation by these factors is unclear, however, MKK7, JNK, and Jun have been implicated in the process by several different approaches [Wolter et al., 2002; Black et al., 2004].

An analysis of ATX mRNA levels using a publicly available transcriptional profiling database (http://www.gnf.org/cancer/epican) indicates that levels of ATX mRNA are low in adult epithelial cells derived from a variety of tissues [Su et al., 2001]. As compared to the levels in normal epithelium, ATX mRNA levels are remarkably increased up to several thousand folds in kidney (renal cell carcinoma) tumors suggesting an important role in this tumor lineage (Fig. 3, Table I). Suppressed subtractive hybridization had previously demonstrated over expression of ATX in renal cell carcinoma [Stassar et al., 2001] compatible with these results. Renal cell carcinomas are amongst the most vascular tumors and contain high levels of angiogenic factors suggesting a potential role for LPA-mediated production of angiogenic factors such as VEGF, IL8, and IL6 in this

disease [Takahashi et al., 1994]. This is compatible with our observations that LPA is a potent induced of angiogenic factors including VEGF, IL8, and IL6 and with the high levels of VEGF present in ovarian cancer ascites [Zebrowski et al., 1999; Hu et al., 2001; Schwartz et al., 2001; Fang et al., 2003]. According to the SAGE database, ATX mRNA levels are low to undetectable in normal kidney suggesting that the increased levels of autotaxin mRNA in renal cell carcinoma represent a novel acquisition of expression. ATX mRNA levels are more modestly but still markedly (100 fold) increased in a broad spectrum of cancers, including liver, gastric, ovary, lung, liver, prostate, and bowel (Fig. 3 and Table I). The modestly elevated levels in ovary and prostate may contribute to the autocrine LPA loops present in both of these tumors [Fang et al., 2002; Mills et al., 2002; Xie et al., 2002; Feng et al., 2003; Mills and Moolenaar, 2003]. It is important to note that within a given tumor type expression levels vary markedly with only modest or no increases in some tumors coupled with remarkable increases at least hundred fold in others. Transcriptional profiling data from Stanford [Schaner et al., 2003] and our own group indicates at least a 2 fold increase with a range of 1–12 in more that half of ovarian cancers as compared to normal ovarian epithelial cells. The discrepancy in relative increases between data sets likely arises from the low levels of ATX mRNA in normal tissues and need to use this as a comparator. Nevertheless, this confirms that ATX mRNA levels are increased in a number of cancer lineages.

We thus assessed ATX levels in a number of tumor cell lines using quantitative PCR. As indicated in Figure 4, ATX mRNA levels vary markedly among tumor cell lines [IOSE29 is used to represent a normal, non-transformed cell line, Auersperg et al., 1999]. However, compatible with the transcriptional profiling data, ATX levels were markedly elevated in a subset of tumor cell lines, particularly glioma and renal cell carcinomas. Even in these two cell lineages, some tumors show limited increases in ATX levels. More modest increases are observed in ovarian cancer cell lines with only limited changes in a subset of breast lines.

If the marked increase in mRNA levels of ATX in glioma and renal cell carcinoma (Figs. 3 and 4) results in release of active enzyme and diffusion into the plasma, ATX levels and ATX activity
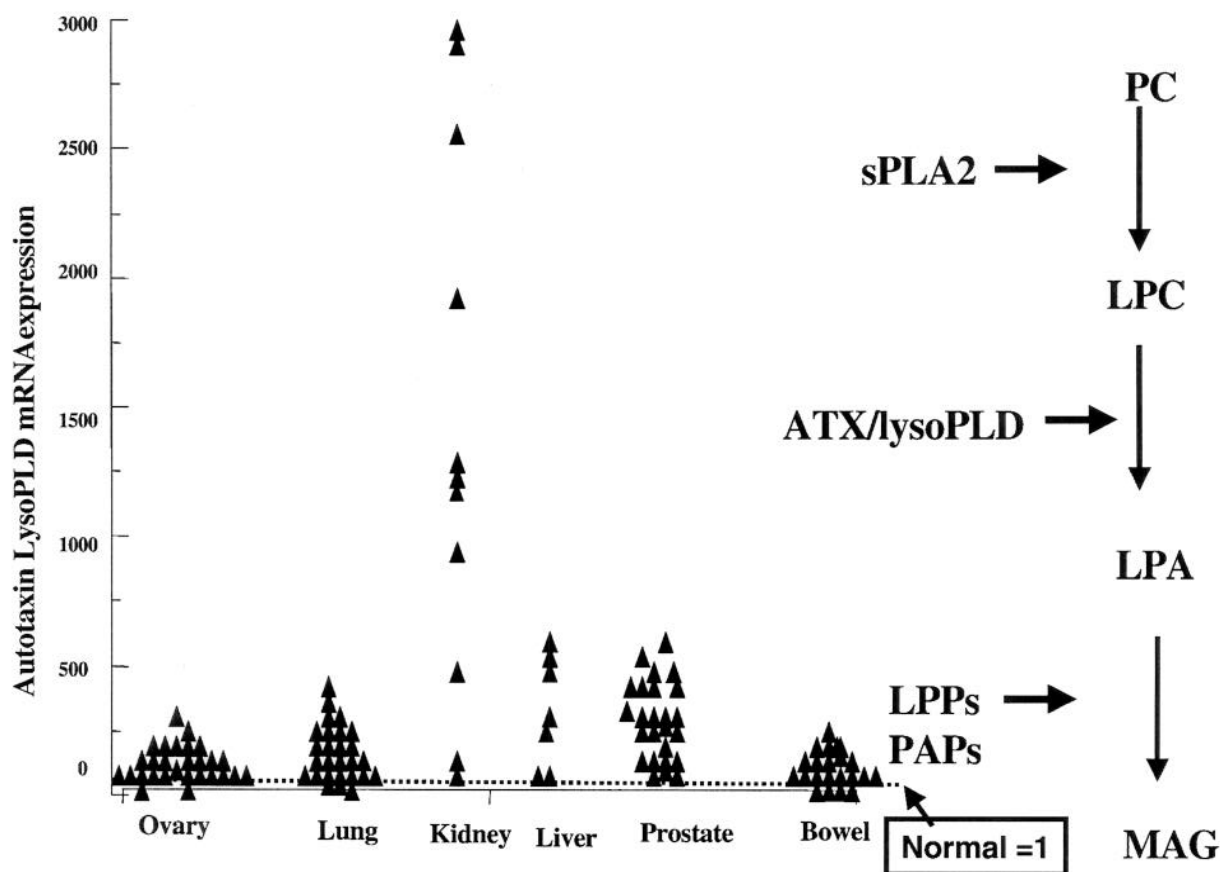
Fig. 3. ATX/LysoPLD mRNA levels are increased in cancer using a publicly available database of Affymetrix U95 arrays (http://www.gnf.org/cancer/epican 74), autotaxin mRNA levels are shown to be markedly aberrant in a number of tumor types. Data is expressed for each tumor relative to control values based on 36 normal adult epithelial tissues. As indicated in the figure, a number of tumors from each cell lineage express elevated levels of autotaxin mRNA.

could be markedly increased in a number of tumor types. To rapidly quantify the lysoPLD activity of ATX in patient samples, a novel fluorogenic assay was developed using the principles developed earlier for PLA activity measurement [Feng et al., 2002]. We thus evaluated ATX enzyme activity assay based on a quenched dye approach detecting the release of fluorescent ethanolamine from its quencher on a synthetic LPE [Drees et al., 2003; see Fig. 5 legend]. OVCAR3 express modest levels of ATX (Fig. 4) and also produces low levels of LPA in culture [Eder et al., 2000], compatible with the basal level of ATX activity. As indicated in Figure 6, cell supernatants from OVCAR3 cells induced a time dependent increase in fluorescence due to unquenching of the fluorescent dye. Further, fetal bovine serum, which contains high levels of ATX and supernatants from lysoPLD/ATX transfected OVCAR3 cells demo-

nstrated increased lysoPLD activity (Fig. 6). This assay was used to assess the levels of ATX activity in plasma and sera samples from controls and patients. As indicated in Figure 7, levels of ATX activity in ovarian cancer ascites were markedly elevated as compared to plasma and sera. There was a statistically significant increase in ATX/lysoPLD activity in the plasma of patients at diagnosis with ovarian cancer and glioma but not in patients with breast cancer similar to the ATX mRNA levels in tumors and cell lines from these cancers (Fig. 7). Similar results were obtained for serum, which had modestly elevated activity as compared to plasma suggesting that ATX is activated (derepressed) or released from blood cells during the coagulation process. However, the increase in ATX activity in serum and plasma from cancer patients were modest as compared to normal controls and exhibited significant overlap

TABLE I. Analysis of Gene Expression Profiles of *LPP* Genes in Different Tumor Samples From Published Microarray Hybridization Data

Enzyme	Type of cancer									
	Ovarian	Breast	Colon	Prostate	Kidney	Pancreas	Bladder	Liver	Gastric	Lung
LPP-1	0.2 + 0.1	0.4 + 0.1	0.3 + 0.1	6.0 + 2.15	2.6 + 1.3	0.7 + 0.2	1.6 + 0.8	1.0 + 0.3	0.4 + 0.4	0.5 + 0.3
LPP-2	1.1 + 1.2	2.8 + 1.5	3.1 + 1.0	1.0 + 1.0	0.4 + 0.8	3.7 + 1.3	2.0 + 1.1	0.6 + 1.6	2.1 + 1.8	2.2 + 1.0
LPP-3	1.0 + 1.1	0.6 + 0.5	0.5 + 0.4	2.9 + 1.2	4.7 + 2.6	0.9 + 0.4	1.2 + 0.8	1.8 + 2.3	0.2 + 0.4	1.1 + 0.4
ATX	34.3 + 32	27.7 + 27	20.7 + 17	73.8 + 46	689 + 493	61.4 + 37	58.5 + 59	121 + 95	24.6 + 35	56.6 + 43.5

Affymetrix U95 array hybridization data are publicly available on 10 different primary human cancers and normal epithelial samples (http://www.gnf.org/cancer/epican 74). The control samples consisted of 36 normal adult epithelial tissues. To allow comparison across multiple tumor types, the mRNA expression data of each gene was normalized to the expression detected in normal epithelial cells. The data is expressed as average of expression ± standard deviation relative to normal epithelial cells. The sample numbers varied between from 6 to 28 for different tumor types.

with ATX levels in normal individuals (Fig. 7) suggesting that an analysis of ATX activity, at least by this approach, in serum or plasma would not provide clinically relevant data. This is in concordance with an earlier report on ovarian cancer sera that failed to detect clinically relevant increases in lysoPLD activity [Tokumura et al., 2002d].

Both LPA and ATX, through LPA production, have been implicated in the activation, proliferation and survival of several cancer cell lineages, but have not been assessed in the pathophysiology of renal cell carcinoma. As indicated above, the ability of ATX and LPA to induce the production of angiogenic factors combined with the high level of these factors produced by renal cell carcinoma cells suggested that ATX and LPA may be critical players in renal cell carcinoma. Given the high level of ATX mRNA in renal cell carcinomas and in the UM-RC-7 renal cell carcinoma cell line (Fig. 4), we explored the effects of decreasing ATX levels with siRNA on cellular signaling and on cell cycle progression and apoptosis in UM-RC-7 cells. As indicated in Figure 8, siRNA specific to autotaxin markedly downregulates ATX expression, an effect that persists for at least 72 h. Compatible with decreased endogenous LPA production, activated MAPK and AKT levels decreased, albeit with greater decreases at later time points (Fig. 8) (total AKT and total MAPK serve as loading controls indicating that equal amounts of proteins were assessed). The delayed decrease in cell signaling as compared to ATX levels may reflect persistence of extracellular ATX or of LPA in supernatants. Nevertheless, siRNA effectively downregulates ATX expression in UM-RC-7 cells and down regulation of ATX is associated with a marked decrease in cell signaling.

We thus explored the effects of down regulation of ATX on cell cycle progression and apoptosis as indicated by cells with a hypodiploid DNA content. In UM-RC-7 cells in both the presence and absence of sera, down regulation of ATX, resulted in an arrest of cells in S phase with an accumulation of apoptotic cells as indicated by an increase in hypodiploid (sub G0/G1) cells (Fig. 9). Similar increases in apoptosis and accumulation of cells in S phase were noted in both glioma and breast cancer cells (not presented). The marked accumulation of cells in S phase was unexpected and suggests activation of the intra-S cell cycle checkpoint, an enigmatic
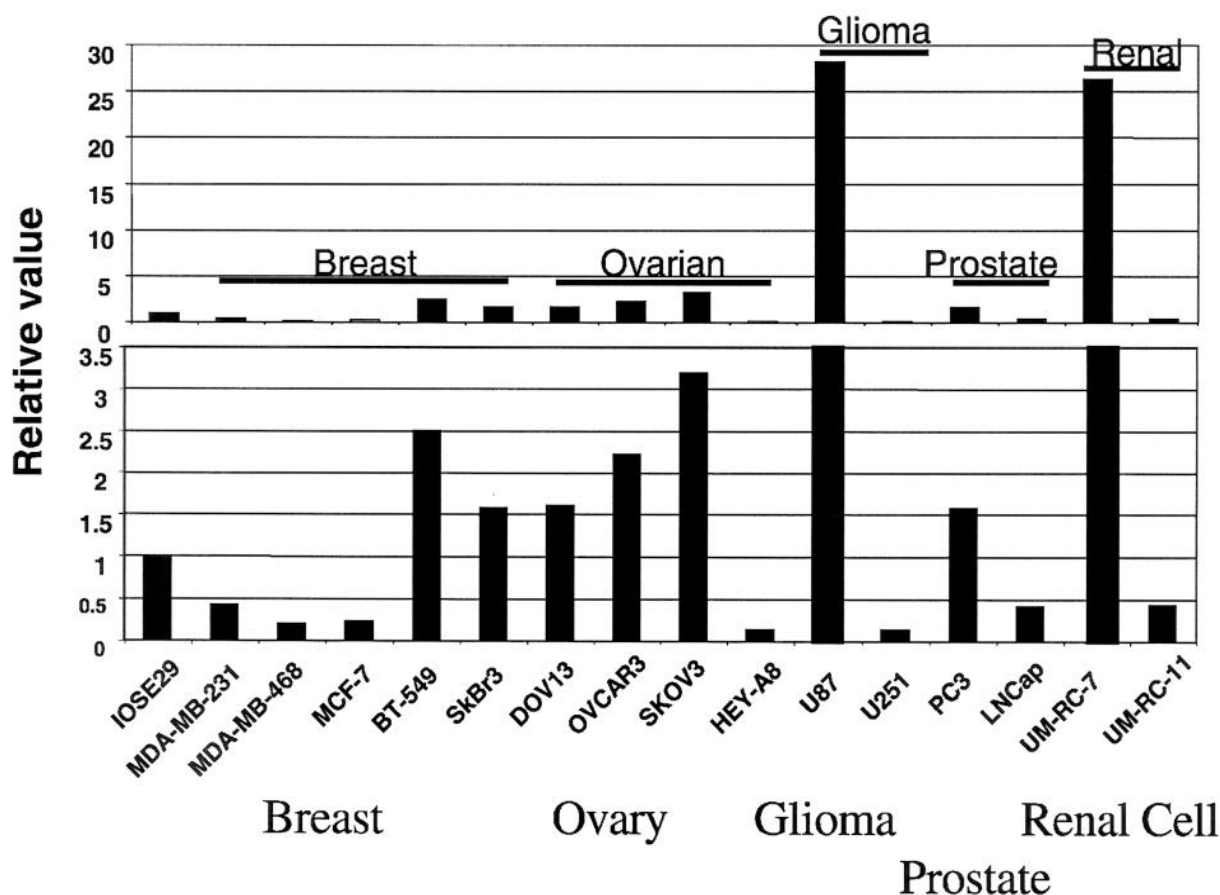
Fig. 4. LysoPLD/ATX RNA levels in tumor cell lines Quantitative PCR was performed on a series of breast, ovarian, glioma, prostate and renal cancer cell lines. Data is normalized to IOSE 29, a SV40 T antigen semi-immortalized cell line that is set to 1. As indicated in the figure, the levels of ATX/lysoPLD mRNA are variable between cell lines including within specific tumor types. U87, a glioma cell line, and UM-RC-7, a renal cell line, express markedly elevated levels of ATX/lysoPLD RNA levels.

check point previously implicated in repair of DNA damage. We have previously overexpressed LPA phosphatases (LPPs) in ovarian cancer cells [see below Tanyi et al., 2003a,b], which increase LPA hydrolysis and should thus have similar effects to autotaxin siRNA that decreases LPA production. Indeed, overexpression of LPPs resulted in an accumulation, albeit more modest that ATX siRNA, in cells in S phase as well as increased apoptosis. The mechanism leading to an increased in cells in S phase is unknown and the topic of ongoing studies.

Taken together, ATX mRNA levels are markedly elevated in a number of cancer cell lineages. Although ATX activity is increased in the plasma and serum from patients with particular types of cancer, the increases are modest and unlikely to be clinically or diagnostically relevant. However, increased autotaxin

activity at the tumor cell interstitial fluid interface is likely to result in increased production of LPA. Indeed, in ovarian cancer patients, genes that are co-regulated with ATX comprise an LPA-dependent transcriptome (not presented), potentially providing a method to identify LPA-dependent tumors likely to be responsive to manipulation of ATX activity or LPA expression. In tumor cell lines over expressing ATX, silencing of ATX with siRNA to ATX results in decreased transmembrane signaling, S phase arrest, and accumulation of apoptotic cells. This combined with previous data implicating ATX in tumor proliferation, motility, aggressiveness, and metastases validates autotaxin and by implication LPA production and action as a target for tumor therapy. As ATX functions as an extracellular enzyme, it is highly accessible for inhibition by antibodies, peptides, pseudo-substrates, or small molecule drugs.
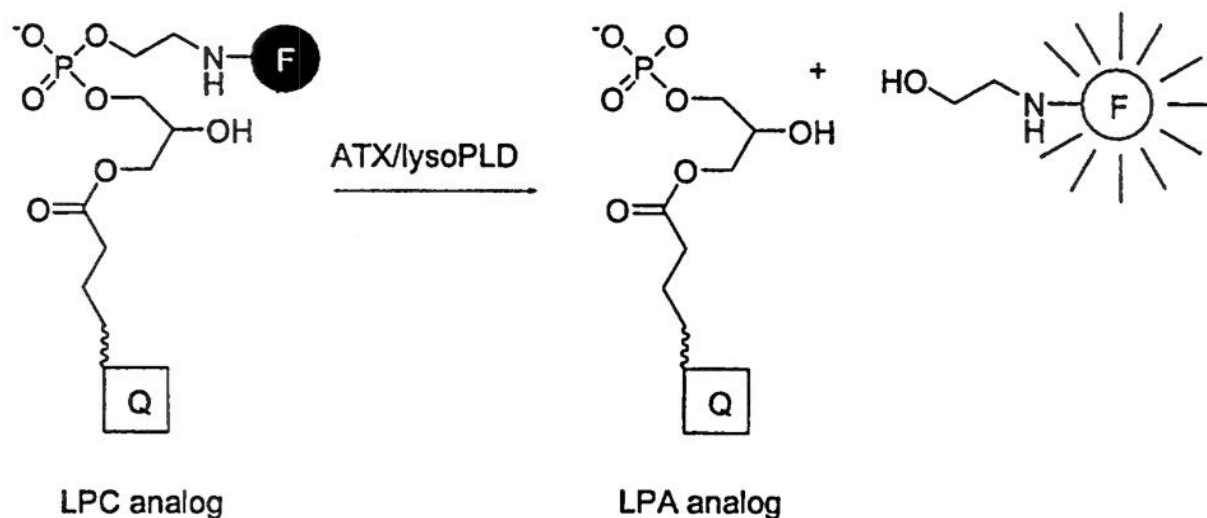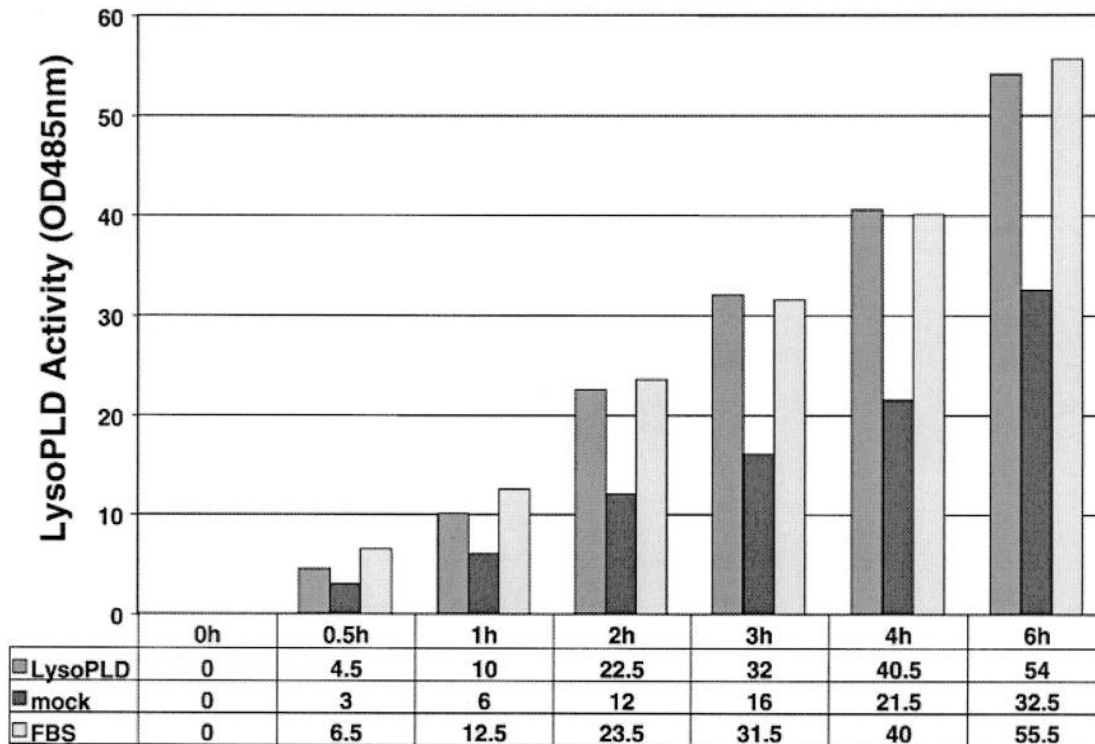
LPC analog   **LPA analog**

Fig. 5. Quenched fluorescence lysoPLD enzyme assay. A quenched cleavable substrate assay similar that developed for PLA2 [Feng et al., 2002] was established for LysoPLD. A doubly labeled substrate analog based on LPE was synthesized containing fluor and quencher moieties. The close intramolecular proximity of the quencher and fluorescent groups results in effective quenching of fluorescence by energy transfer in the substrate. Upon digestion by lysoPLD, the ethanolamine group containing the fluor is released, energy transfer is interrupted, and fluorescence increases as a function of the extent of lipase activity.

LPA Degradation

LPA can be removed by cleavage of the acyl chain by phospholipases, reacylation by LPAAT or endophilin, or by hydrolysis of the phosphate by lysophosphatidic acid phosphatases [LPP-1, -2, -3 also know and PAPs or PA phosphatases, Brindley et al., 2002; Mills et al., 2002; Tanyi et al., 2003a,b; see studies in this review]. LPPs are hexahelical transmembrane lysolipid phosphatases with an extracellular catalytic site, suggesting a role in hydrolysis of extracellular LPA. Although different LPPs demonstrate modest selectivity for lysophospholipid isoforms, each of the LPP isoforms can hydrolyze LPA, S1P or ceramide 1-phosphate and likely other lysophospholipids. They exhibit little activity however toward PA or other diacyl chain lipids. Over 95% of hydrolysis of LPA by cancer cells appears to be due a membrane associated LPP activity suggesting that LPPs are the major method for clearance of LPA in vivo [Imai et al., 2000]. As indicated in Table I (relative levels) and Figure 10 (absolute levels), LPP mRNA levels and in particular LPP-1 mRNA levels demonstrate marked variability in different tumor lineages. For example, LPP levels are elevated in prostate and kidney cancers whereas they are markedly decreased in ovary, lung, and breast cancer. Intriguingly, prostate and renal cell carcinomas, which have high levels of autotaxin mRNA (Table I, Fig. 3)

also exhibit elevated levels of LPP-1 mRNA. Thus there is likely to be an increased production and hydrolysis of LPA in these tumor types with potentially increased information flow through the pathway. What the net effect of overexpression of both ATX and LPP-1 is on LPA production and degradation is remains to be assessed. LPP-1 levels are markedly decreased in ovarian cancer (Table I, Fig. 10), potentially contributing to the elevated levels of LPA present in ascites. We have explored the role of LPPs in ovarian cancer by increasing expression of LPP-1 and determining effects on cellular functions [Tanyi et al., 2003a,b]. In most ovarian cancer cells, overexpression of LPPs is incompatible with cell proliferation and survival. Overexpression of LPP-1 or LPP-3 in SKOV3 cells which have high levels of ATX (Fig. 4) and produce LPA spontaneously [Eder et al., 2000] is tolerated by the cells. Stable LPP overexpression decreases LPA levels, increases LPA hydrolyses, inhibits cell cycle progression resulting in an S phase arrest and accumulation of apoptotic cells and decreases growth subcutaneously and intraperitoneally [Tanyi et al., 2003a,b]. In a typical experiment (Fig. 11), transient overexpression of LPPs markedly decreases colony forming cell activity. Strikingly, this effect is reversed by addition of XY-13 (Fig. 11) and XY-14, two enantiomeric phosphonate analogues of PA [Xu and Prestwich, 2002], that act as potent inhibitors of the

	0h	0.5h	1h	2h	3h	4h	6h
LysoPLD	0	4.5	10	22.5	32	40.5	54
mock	0	3	6	12	16	21.5	32.5
FBS	0	6.5	12.5	23.5	31.5	40	55.5

The culture media from ATX / LysoPLD or control vector transfected OVCAR3, and FBS were subjected to LysoPLD assay. They showed time-dependent increase in activity and an increase in transfected cells.

Fig. 6. Validation of the lysoPLD assay. The quenched cleavable lysoPLD assay described in Figure 1, was validated by incubation with supernatants from lysoPLD and mock transfected OVCAR3 cells (OVCAR3 cells have modest lysoPLD mRNA levels) as well as with fetal bovine serum, which has high levels of lysoPLD [Umezu-Goto et al., 2002]. As indicated in the figure, following background subtraction there is a linear time dependent increase in lysoPLD substrate cleavage.

LPPs [Smyth et al., 2003]. The effect is also reversed by OMPT an LPP-resistant LPA analog [Hasegawa et al., 2003] that shows enantioselective activation of LPA3 [Qian et al., 2003] confirming that the effect of the LPPs is through degradation of LPA (Fig. 11). In support of hydrolysis of LPA as a mechanism of action, overexpression of LPPs in SKOV3 cells resulted in a marked decrease in the proliferation and survival of non-transfected SKOV3 cells, a bystander effect. This extracellular mechanism of action and strong bystander effect is optimistic for potential gene therapy approaches to ovarian cancer, a process being explored by our group.

The combined studies with autotaxin siRNA and overexpression of LPPs provide strong evidence validating the LPA pathway as a

target for therapy in cancer. This suggests that drugs targeting autotaxin, which is relatively specific for LPA production (SPC levels are low extracellularly compared to LPC), or specific LPA receptors or their downstream signaling pathways may have efficacy in the therapy of cancer [Tokumura et al., 2002c; Umezu-Goto et al., 2002; Clair et al., 2003]. As indicated above, LPA and autotaxin have pleiomorphic effects in multiple cell lineages, suggesting that inhibition of the LPA production or action may be toxic. However, knockouts of LPA1 and -2 and indeed combined LPA1 and -2 knockout mice are viable suggesting either that these pathways are not required for normal cellular physiology or that LPA3 and -4 exhibit redundant activities [Yang et al., 2002a]. These questions will be answered through the development
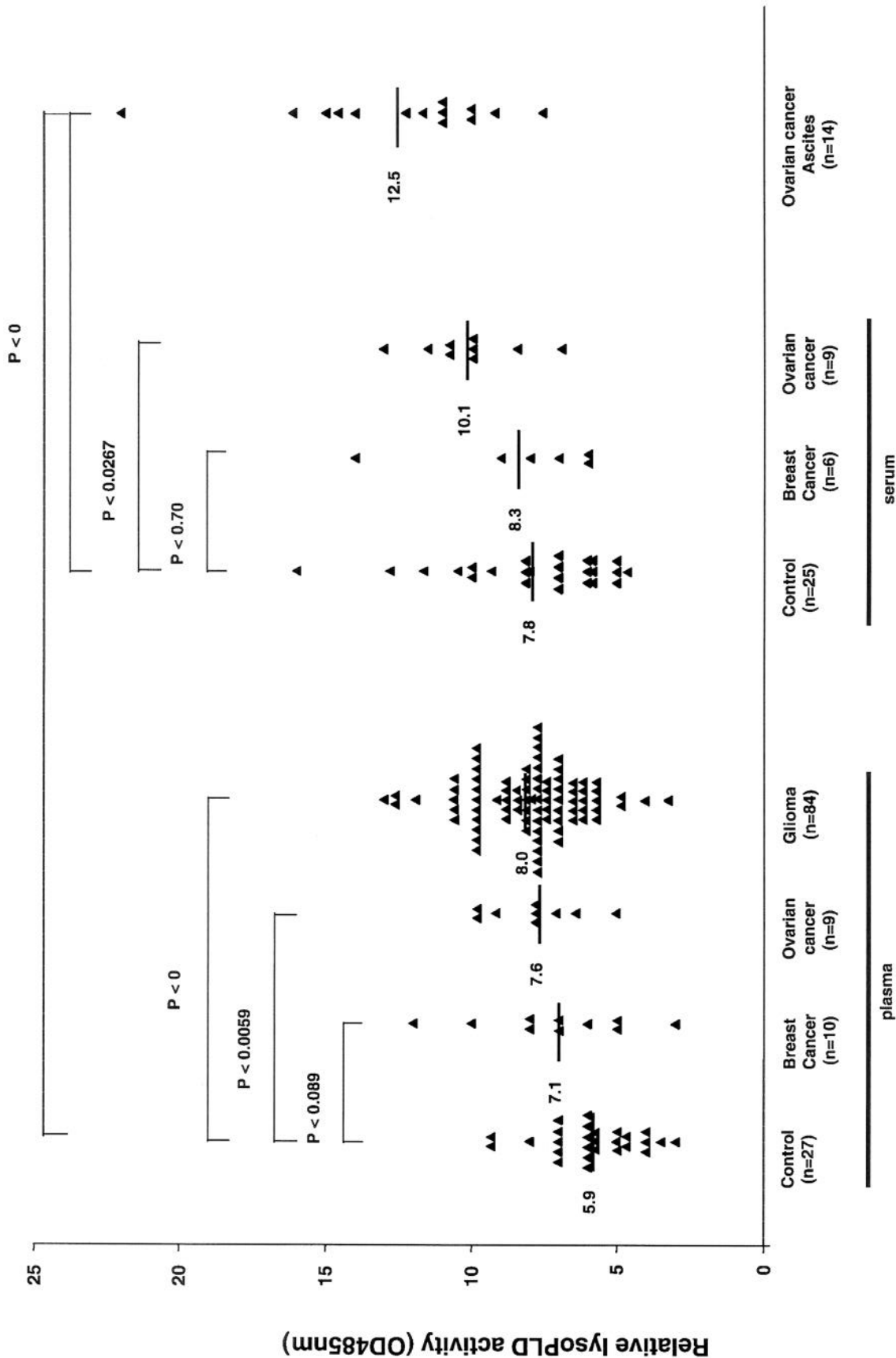
Fig. 7. LysoPLD activity in patient samples using the validated lysoPLD assay, lysoPLD enzyme activity was measured in serum and plasma of patients with glioma, ovarian cancer or breast cancer. Serum and plasma activity was assessed for breast and ovary and ascites levels for ovarian cancer. Mean levels are indicated by a bar for each set. Statistically different results (Student's t-test) are indicated in the figure.
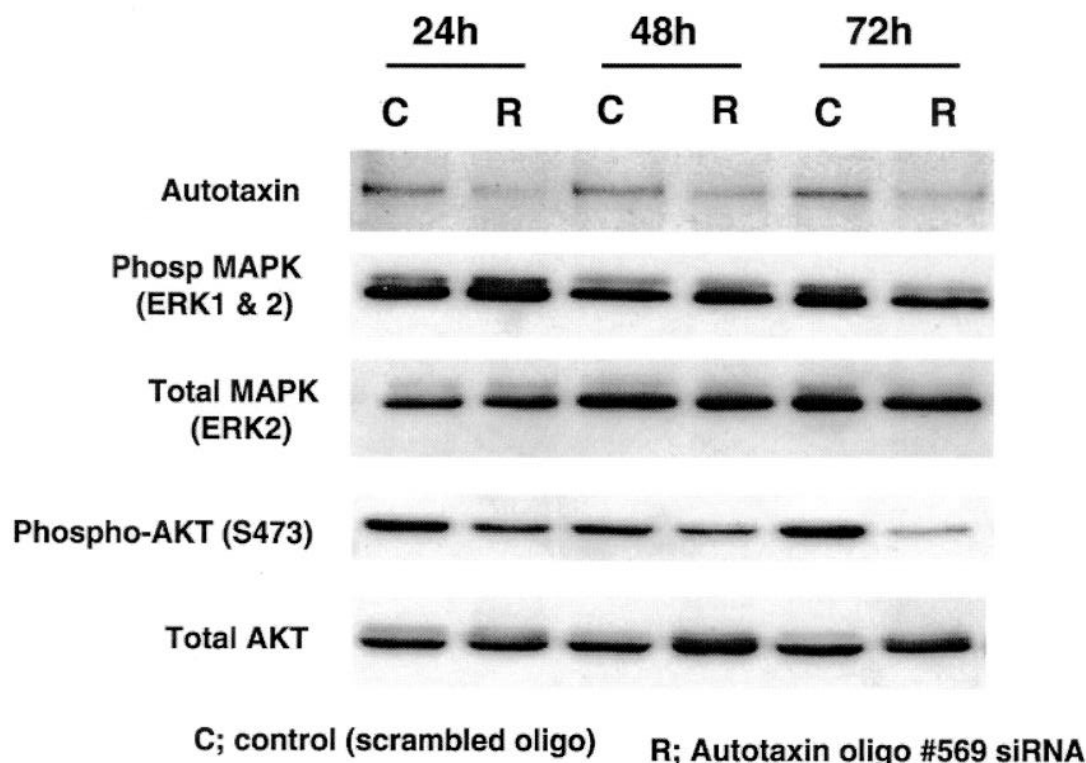
	24h		48h		72h	
	C	R	C	R	C	R

Autotaxin

Phosp MAPK (ERK1 & 2)

Total MAPK (ERK2)

Phospho-AKT (S473)

Total AKT

C; control (scrambled oligo)   R; Autotaxin oligo #569 siRNA

Fig. 8. RNAi to ATX /lysoPLD decreases ATX levels and decreases signaling to AKT and MAPK RNAi specific to autotaxin was introduced into UM-RC-7 renal cell carcinoma cells that overexpress autotaxin (Fig. 4). The RNAi induced a prolonged and significant downregulation of autotaxin protein levels in the cell line. This decrease was associated with decreased phosphorylation of both erk1 and erk2 as well as AKT. There was no decrease in total MAPK or AKT demonstrating equal loading and lack of non-specific effects.

and assessment of small molecule inhibitors and agonists of specific LPA receptors, a process which we have recently reviewed [Feng et al., 2003].

LPA in Ovarian Cancer

Ovarian cancer has been extensively explored as a model for aberrations in LPA production, metabolism and function [Xu et al., 1995, 1998, 2003; Shen et al., 1998, 2001; Westermann et al., 1998; Eder et al., 2000; Xiao et al., 2000, 2001; Hu et al., 2001; Schwartz et al., 2001; Baker et al., 2002; Fang et al., 2002, 2003; Mills et al., 2002; Feng et al., 2003; Luquain et al., 2003a; Mills and Moolenaar, 2003; Sengupta et al., 2003]. Although it is clear that LPA plays a major role in the pathophysiology of ovarian cancer, data is rapidly accumulating [see above Mills and Moolenaar, 2003], implicating LPA in the pathophysiology of multiple different cancers. Nevertheless, due to the wealth of data in ovarian cancer, the remainder of this review will concentrate on the unique aspects of

ovarian cancer and what is known about LPA production, metabolism, and action in ovarian cancer.

LPA levels. It is difficult to determine the levels of LPA and indeed any growth factor in the interstitial fluid of tumors and thus we have been limited in our ability to assess whether cancer cells, in vivo, are stimulated by particular growth factors. The ascitic fluid that accumulates in ovarian cancer patients provides access to the distinctive ovarian cancer microenvironment. The growth factor composition of ascitic fluid reflects growth factor production, degradation and clearance from the local environment and also reflects the growth factor environment of the tumor cell. We have demonstrated that malignant ascites from ovarian cancer patients contain high levels of growth factor activity both in vitro and in vivo [Fang et al., 2002; Mills et al., 2002]. At least a portion of the growth factor activity in ascites can be attributed to the presence of high concentrations of LPA and LPA like molecules present

72h	0% FBS		10% FBS	
	control	siRNA	control	siRNA
Sub G0 / G1	4.27%	9.73%	1.21%	3.92%
G0 / G1	88.34%	58.99%	90.10%	41.95%
S	4.67%	27.10%	5.57%	54.83%
G2 / M	6.98%	13.91%	4.33%	3.22%

Fig. 9. RNAi to ATX induces a S phase arrest and apoptosis. RNAi specific to ATX was introduced into UM-RC-7 renal cell carcinoma cells that overexpress ATX. Seventy-two hours later adherent and floating cells were collected and analyzed for cell cycle and apoptosis (hypodiploid peak) using flow cytometry of propidium iodide labeled cells. RNAi induced a marked accumulation of cells in S phase as well as hypodiploid cells. Similar results were obtained at 48 h.

in ascites (1–80 μM) [Westermann et al., 1998; Eder et al., 2000; Xiao et al., 2001; Fang et al., 2002; Mills et al., 2002; Mills and Moolenaar, 2003; Xu et al., 2003]. Ovarian cancer ascites not only contains consistently elevated levels of LPA, but the LPA present contains a number of unusual forms, which may have novel mechanisms of production or action [Xiao et al., 2001].

Since LPA levels are elevated in ascites of ovarian cancer patients, if LPA migrates to the periphery, there is a potential that LPA levels could be elevated in the plasma of patients providing an early diagnostic marker or marker of disease behavior. Indeed, LPA levels are consistently higher in ascites samples than matched plasma samples suggesting that LPA produced in the peritoneal cavity migrates into the plasma [Eder et al., 2000]. In a preliminary analysis with a limited number of patients and controls, LPA levels were elevated in more than 80% of stage I ovarian cancer patients with a false positive rate of 5% [Xu et al., 1998, 2003]. LPA levels are not elevated in blood samples

from patients with most other cancers, but are increased in patients with myeloma, endometrial cancer, cervical cancer, and renal dialysis [Sasagawa et al., 1998, 1999], all of which can be distinguished from ovarian cancer by medical assessment. Following the original publication, a number of reports have supported or refuted the potential utility of LPA levels in early diagnosis and prognosis of ovarian cancer [Xiao et al., 2000; Shen et al., 2001; Baker et al., 2002; Yoon et al., 2003; Xu et al., 2003]. As plasma contains enzymes required for the production and metabolism of LPA [Xu et al., 1998; Aoki et al., 2002], differences in the results from the groups likely arise from challenges in the collection and handling of plasma to prevent post collection production, metabolism or loss of LPA. An additional complication arises in the analysis of LPA, which requires purification, concentration and multiple handling steps, which could result in artifactual changes in LPA levels. Current studies have focused on total LPA levels and levels of LPA isoforms. It
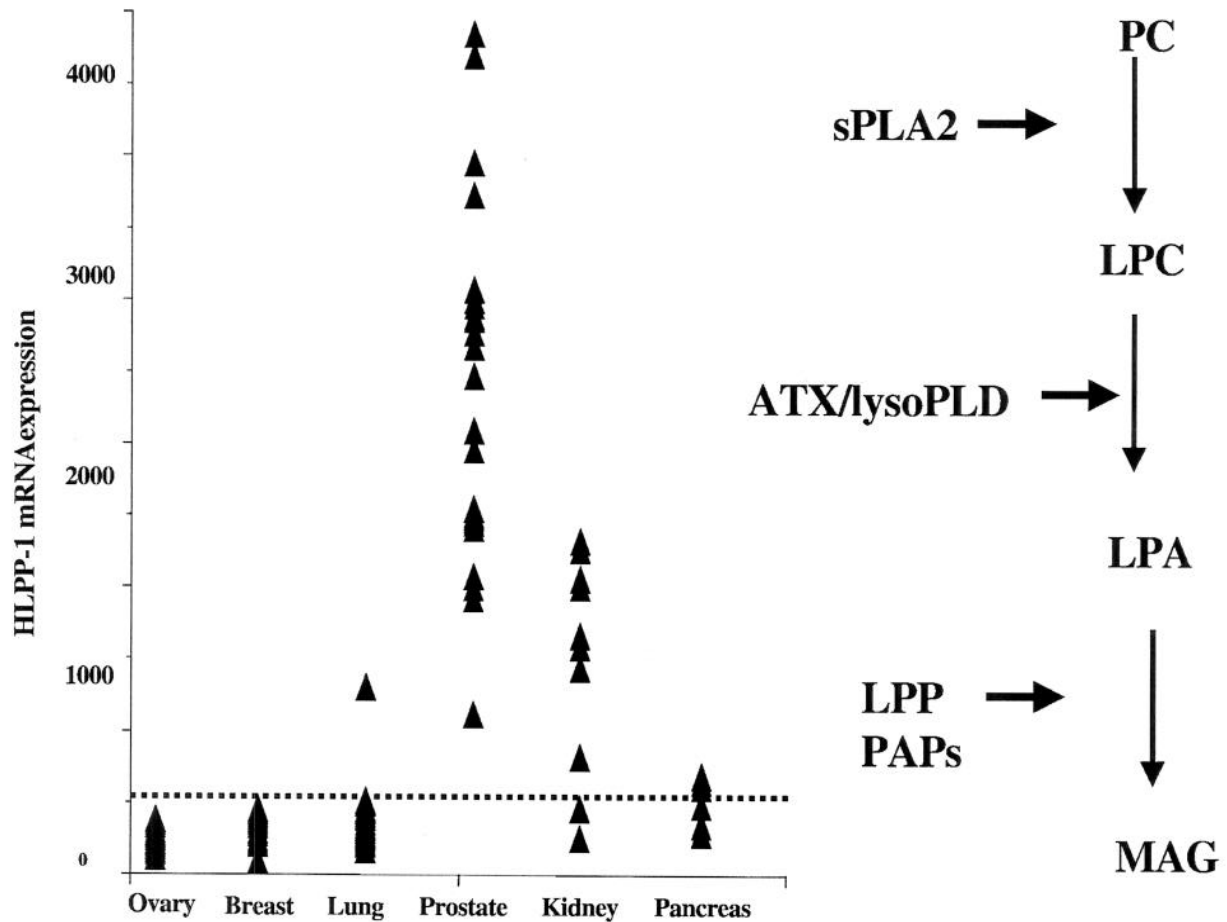
LPP mRNA LEVELS ARE ALTERED IN CANCER

Fig. 10. LPP-1 mRNA levels are altered in cancer using a publicly available database of Affymetrix U95 arrays (http://www.gnf.org/cancer/epican), LPP-1 mRNA levels are shown to be markedly aberrant in a number of tumor types. Data is expressed for each tumor relative to control values based on 36 normal adult epithelial tissues. As indicated in the figure, LPP-1 mRNA levels are elevated in a number of tumor types, and decreased in others.

may, however, be necessary to assess the composition of particular isoforms of LPA or other lysophospholipids and related lipids precursors and breakdown products as part of a lipid profile to develop an algorithm able to detect clinically relevant changes in lipid levels or composition. Such approaches using global analysis of small molecules by mass spectroscopy has been designated metabolomics and a release of this technology as a clinically available "home brew" screening test for ovarian cancer is being proposed for early 2004. A reanalysis of data and follow up data from the original study describing the use of pattern recognition of small molecules by SELDI-TOF mass spectroscopy as a potential approach to

detect ovarian cancer [Petricoin et al., 2002], suggested that a number of different molecules in the range of 200–800 Da had discriminatory power for the detection of ovarian cancer. A recent follow up study, indicates that the majority of these molecules are carried in the blood stream bound to albumin and other carrier proteins [Mehta et al., 2004]. LPA associates with albumin as well as with other carrier proteins such as gelsolin [Goetzl et al., 2000] suggesting that it may be in the carrier protein compartment. Combined with the observation that fatty acyl chains (200–300 Da), monoacylglycerols (300–400 Da), lysophospholipids (400–600 Da), and phospholipids (700–900 Da) fall into this range, it is possible that a
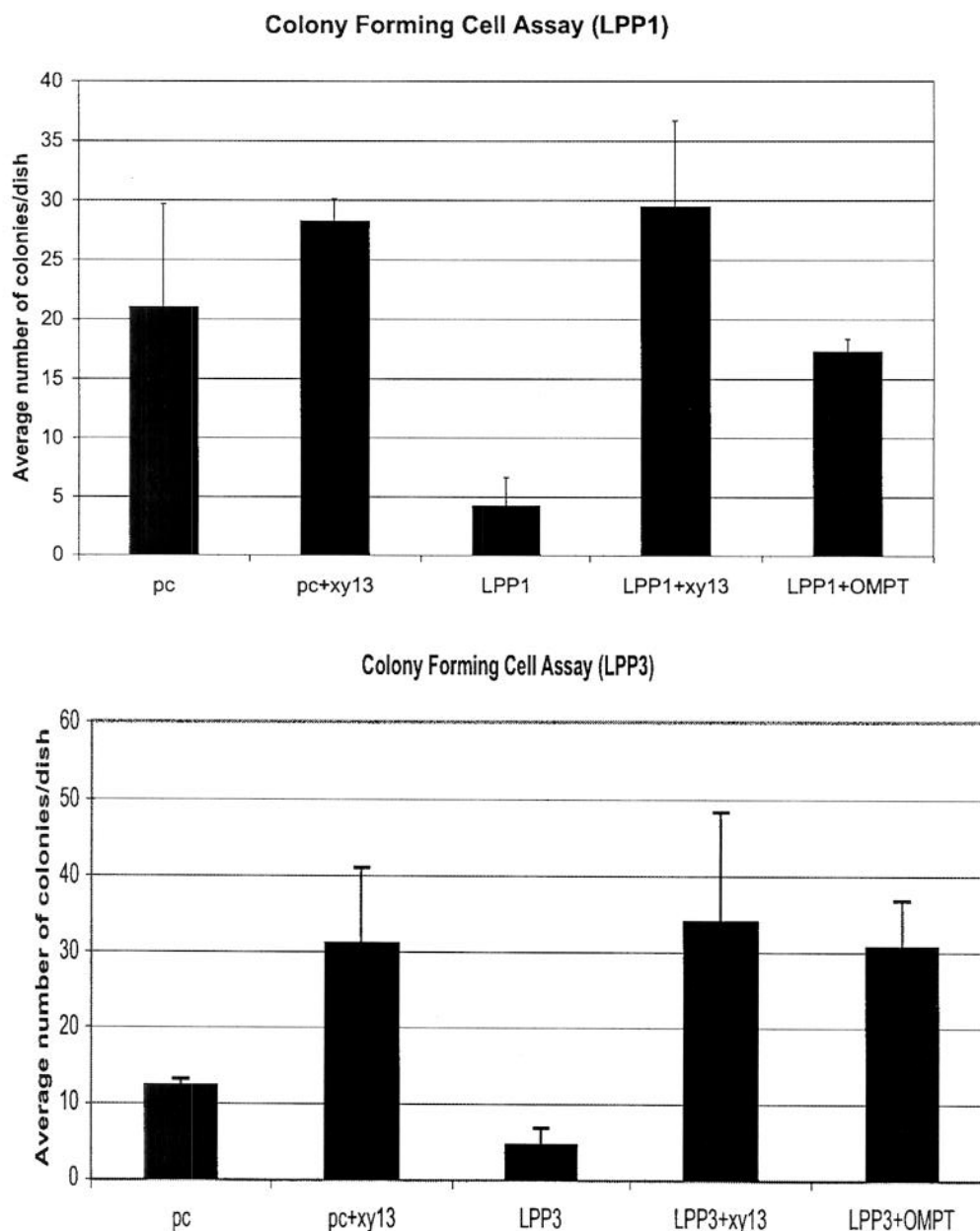
Colony Forming Cell Assay (LPP1)

Colony Forming Cell Assay (LPP3)

Fig. 11. LPP-1 and LPP-3 decrease colony forming cell activity of ovarian cancer cells through degradation of LPA. As indicated in Figure 10, LPP-1 levels are decreased in ovarian cancer (85). Transient transfection with LPP-1 or LPP-3 induces a marked decrease in colony forming cell activity in SKOV3 ovarian cancer cells, which express high levels of LysoPLD and produce LPA constitutively. Transfected cells were incubated with XY13 that inhibits LPPs and with OMPT, a LPP resistant LPA analog. Both XY-13 and OMPT reversed the effects of LPP on colony forming cell activity demonstrating that the effects of LPP-1 and LPP-3 were due to degradation of LPA.

global analysis of lipid patterns could prove diagnostic for particular cancers. We have demonstrated that model lipids bind with high affinity to the matrices used in SELDI, are excited by the laser and are easily visualized by SELDI-TOF. A prospective clinical trial to evaluate the utility of assessment of patterns of lipids in plasma measured by ESI (metabo-

lomics), SELDI- or MALDI-TOF or other approaches as an ovarian cancer marker is clearly warranted.

Until recently the mechanisms resulting in high levels of LPA present in ovarian cancer ascites remained elusive. However, as indicated in Figure 12, a number of enzymes producing LPA including sPLA2 [Ben-Shlomo et al., 1997]
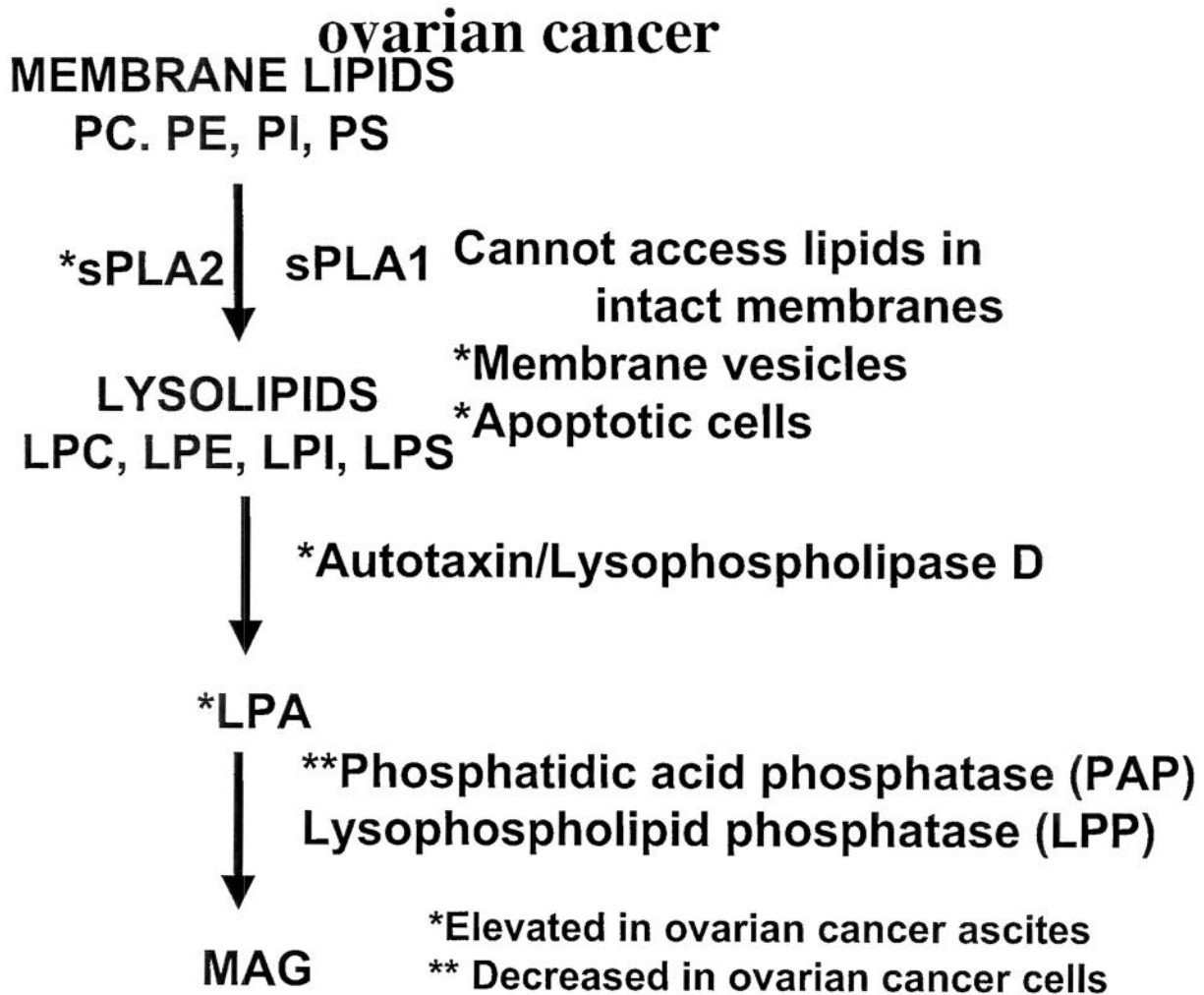
ovarian cancer

MEMBRANE LIPIDS
PC. PE, PI, PS

*sPLA2 | sPLA1   Cannot access lipids in
                  intact membranes
               *Membrane vesicles

LYSOLIPIDS   *Apoptotic cells
LPC, LPE, LPI, LPS

*Autotaxin/Lysophospholipase D

*LPA

**Phosphatidic acid phosphatase (PAP)
Lysophospholipid phosphatase (LPP)

            *Elevated in ovarian cancer ascites
MAG   ** Decreased in ovarian cancer cells

Fig. 12. Aberrations in lipid metabolism in ovarian cancer points of aberrations in lipid metabolism in ovarian cancer are indicated based on the major pathway of extracellular LPA production.

and autotaxin are increased in ovarian cancer patients (Fig. 3), and as noted above, LPP isoforms are decreased (Fig. 10) [Tanyi et al., 2003a,b]. Like many cancers, the number of apoptotic cells in ovarian cancers is high and membranes are accessible to the action of sPLA2 potentially resulting in increased production of substrates for ATX [Fourcade et al., 1995]. Ascites also contains markedly elevated levels of vesicles that are also accessible to sPLA2 [Ginestra et al., 1999; Andre, 2002]. Furthermore, ovarian cancer cells both constitutively and inducibly produce LPA [Shen et al., 1998; Eder et al., 2000; Sengupta et al., 2003]. As patients may have kilograms of tumor present when diagnosed, even a modest increment in production or decreased metabolism of LPA by ovarian cancer cells could result in marked accumulation of LPA in ascites. Indeed,

ascites itself can contain up to 10^9 tumor cells per milliliter, which could result in release of high levels of LPA. Thus, there is a suite of aberrations in ovarian cancer that potentially contributes to the accumulation of LPA at the tumor interface and in ascites. Taken together, the data suggest that the increased levels of LPA in ovarian cancer ascites reflects both increased production and decreased hydrolysis.

LPA is not produced at significant levels by normal ovarian epithelial cells, whereas ovarian cancer cells both constitutively and inducibly produce elevated levels of LPA in response to phorbol esters, laminin and LPA itself [Shen et al., 1998; Eder et al., 2000; Sengupta et al., 2003], suggesting that ovarian cancer cells which present at great numbers in the peritoneal cavity could contribute to the elevated levels. The ability of ovarian cancer cells to

produce and respond to LPA suggests that ovarian cancer cells are likely regulated by autocrine LPA loops. A recent report confirms the presence of an autocrine LPA loop in prostate cancer [Xie et al., 2002] suggesting that LPA autocrine loops may contribute to the pathophysiology of multiple cancers.

In view of the higher levels of LPA in ascites from ovarian cancer patients and the ability of LPA to activate the pathways mediating LPA production, we assessed basal and LPA-induced LPA production by ovarian cancer cells. We found that ovarian cancer cells, in contrast to normal ovarian epithelial cells or breast cancer cells, produce LPA either constitutively or in response to LPA [Eder et al., 2000]. Both constitutive and LPA-induced LPA production exhibited PLD-dependent and -independent components. LPA has been demonstrated to activate PLD in a number of systems [van der Bend et al., 1992; Kam and Exton, 2004]. PLD can alter membrane composition and in particular induce production of membrane vesicles [Morgan et al., 1997; Kinkaid et al., 1998]. Indeed, ascites contains high levels of vesicles potentially due to PLD activation [Ginestra et al., 1999; Andre, 2002]. Constitutive LPA production was primarily dependent on group IB (pancreatic) sPLA2 and on cPLA2 and/or iPLA2, whereas LPA-induced LPA production was dependent on both group IB (pancreatic) and group IIA (synovial) sPLA2, but not cPLA2 or iPLA2 suggesting that LPA induces extracellular LPA production [Eder et al., 2000]. LPA is a potent activator of increases in cytosolic calcium and of MAPKs in ovarian cancer cells and both increases in cytosolic calcium and MAPK activity activate cPLA2 potentially contributing to the increases in LPA production. In contrast to LPA-induced LPA production, PMA-induced LPA production is dependent on cPLA2 or iPLA2 indicating effects on intracellular LPA production [Shen et al., 1998]. Laminin-induced LPA production by ovarian cancer cells is dependent on beta1 integrins and iPLA2 similar to PMA-induced LPA production [Sengupta et al., 2003]. Nucleotide agonists acting at the P2Y4 purinergic receptors on ovarian cancer cells also induce LPA production. Nucleotide induced LPA production is associated with an increase in PLD activity and PLD augments LPA production suggestion that it could be a major contributor to LPA production by ovarian cancers [Luquain et al., 2003b]. Ovarian cancer

cells secrete PLA2. Indeed the increased concentration of vesicles in ascites that are accessible to sPLA could contribute to LPA production either by conversion of PA to LPA or through the production of LPC and other autotaxin substrates.

Aberrations in LPA Function in Ovarian Cancer

As noted above, the production of LPA is aberrant in ovarian cancer cells due to changes in the levels and activity of multiple different enzymes resulting in the accumulation of high concentrations of LPA in ascites fluid and likely in the interstitial space in the tumor. This accumulation of LPA would be sufficient to play a major role in the pathophysiology of ovarian cancer. However, the effects of the high levels of LPA present in ovarian cancer patients are amplified by aberrations in the response of ovarian cancer cells to LPA. The expression of LPA receptors on ovarian cancer cells is markedly different from that on normal ovarian epithelial cells resulting in increased responsiveness to LPA [Goetzl et al., 1999; Fang et al., 2002; Mills et al., 2002; Feng et al., 2003]. Further, unusual splice variants of LPA receptors, selectively linking to processes such as production of neovascularizing factors, may be present in ovarian cancer cells [Huang et al., 2004]. Finally, additional genetic aberrations in ovarian cancer cells such as amplification and mutation of multiple components of the phosphatidylinositol 3 kinase pathway sensitize ovarian cancer cells to downstream signaling effects of LPA [Fang et al., 2002; Mills et al., 2002; Feng et al., 2003]. As for changes in LPA production and metabolism, the genetic mechanisms underlying these processes remain to be determined. Nevertheless, taken together, LPA production or action appears well justified as a target for therapy in ovarian cancer.

Although freshly isolated ovarian cancer cell preparations and ovarian cancer cell lines are consistently responsive to LPA, normal ovarian surface epithelial cells (OSE) do not demonstrate significant responses to LPA [Goetzl et al., 1999; Eder et al., 2000; Fang et al., 2002; Mills et al., 2002; Feng et al., 2003]. In contrast, OSE demonstrate marked responses to S1P, whereas ovarian cancer cells demonstrate more limited responses to S1P [Goetzl et al., 1999]. Consistent with the shift from S1P-dependence to LPA-dependence, ovarian cancer cell lines express markedly increased levels of mRNA

and protein for the LPA2 and -3 receptors and decreased levels of mRNA and protein for S1P receptors [Goetzl et al., 1999] as compared to OSE which have very low levels of LPA2 and -3. There are no consistent changes in levels of LPA1 receptors between OSE and ovarian cancer cell lines. However, LPA1 may function as a negative LPA receptor, as overexpression of LPA1 results in decreased proliferation as a consequence of increased apoptosis [Furui et al., 1999]. Analysis of ovarian cancer cells directly from the patient paints a similar picture in that LPA1 levels are not different from those in normal epithelium and LPA2 and -3 are each elevated in approximately 40% of tumors with a significant overlap resulting in increased expression of LPA receptors in approximately 60% of ovarian cancers [Fang et al., 2002]. Thus acquisition of expression of LPA2 and -3 during transformation leads to increased responses to LPA potentially contributing to the pathophysiology of ovarian cancer. LPA4 receptors are reported to expressed in ovary, however, the initial report did not indicate whether this was in stroma or epithelium [Noguchi et al., 2003]. As assessed by QPCR, ovarian cancer cells have low to absent levels of LPA4.

Ovarian cancer cells contain an unusual variant of LPA2 with an intracellular extension [Huang et al., 2004]. This extension destroys the PDZ binding site in LPA2 that may have important functional consequences. The variant exhibits increased ability to link to production of active VEGF, an important permeability and neovascularizing factor [Hu et al., 2001; Huang et al., 2004]. LPA couples efficiently to VEGF production potentially contributing to the elevated VEGF levels in ovarian cancer ascites [Zebrowski et al., 1999; Hu et al., 2001]. Further, although LPA1 and -3 appear able to couple to the production of IL8 and IL6 neovasularization factors, LPA2 appears to most efficient in mediating production of these factors [Schwartz et al., 2001; Fang et al., 2003]. As IL8 production is dependent on NfκB mediated activation of the IL8 promoter, it appears likely that LPA2 couples most efficiently to activation of NfκB [Fang et al., 2003]. In ovarian cancer, VEGF appears to play a major role in the production of ascites whereas IL8 production appears to regulate aggressiveness and potentially neovascularization. The effects of the increased ability of LPA2 to couple to these pathways suggest that inhibitors targeting the LPA2 receptor may selectively alter neovascularization.

Expression of the ovarian cancer specific LPA2 variant in the stroma (the promoter used does not express or is expressed at low levels in the epithelial cells of the ovary, the most frequent precursor for ovarian cancer) of transgenic murine ovaries results in constitutive production of active VEGF-A again supportive a selective role of LPA2 in production of neovascularizing factors [Huang et al., 2004]. In addition expression of the ovarian cancer specific LPA2 variant increases the production of urokinase (uPA), compatible with previous reports showing that these factors are regulated by LPA in ovarian cancer cells [Pustilnik et al., 1999; Huang et al., 2004]. This was accompanied by increased expression of VEGF receptors and decreased production of type 2 PA inhibitor. Together, this suggests that activation of the LPA2 receptor aberrantly expressed in ovarian cancer cells may contribute to neovascularization and also result in ovarian cancer cells being responsive to VEGF. If similar processes affect other neovascularizing factors such as IL6 and IL8 activation of LPA receptors may stimulate a number of aberrant autocrine loops.

LPA appears to activate a number of additional feed-forward autocrine signaling loops. For example, LPA can induce the production of endothelin, a potent activator of ovarian cancer cells [Chua et al., 1998]. Indeed, endothelin receptor isoforms appear to be aberrantly expressed in ovarian cancer. Alternatively, the effects of LPA on ovarian cancer cells may be due to LPA-induced production or processing of growth factors, which in turn activate cognate cell surface receptors. As described above, LPA induces the activation of a number of tyrosine kinase linked growth factor receptors including multiple members of the human EGF receptor (HER) family, and the platelet-derived growth factor receptor. LPA-induced processing of HB-EGF and PDGF, which, in turn, activates members of the HER family and PDGFR respectively. LPA also induces activation of Src either through activation of tyrosine kinase linked receptors or through other as yet unclear mechanisms. As src and EGFR family members are overexpressed in ovarian cancers, this may amplify the effects of LPA. Indeed, we have demonstrated that LPA induces tyrosine-phosphorylation of multiple members of the HER family and of Src in ovarian cancer cells,

compatible with LPA inducing proliferation through the increased production or action of other growth factors which activate members of the HER or PDGFR family [Xu et al., 1995; Fang et al., 2002; Mills et al., 2002; Feng et al., 2003]. Nevertheless, the relative role of the direct and indirect activation of ovarian cancer cells by LPA in the pathophysiology of ovarian cancer remains to be elucidated.

Ovarian cancer cells may also have aberrant responses to other lysophospholipids. As indicated above, there are aberrations in the expression of both LPA and S1P receptors that could alter the responsiveness of ovarian cancer cells [Goetzl et al., 1999]. In at least one model system, S1P can transdominantly inhibit responses to LPA suggesting that the shift in receptor selectivity from S1P in normal ovarian epithelial cells to LPA in ovarian cancer cells may have functional consequences in addition to the effects of increased LPA receptor levels [Clair et al., 2003]. The OGR1 orphan GPCR, which is overexpressed in some ovarian cancer cell lines, has been demonstrated to be a specific receptor for SPC [Xu et al., 2003]. SPC increases cytosolic calcium in ovarian cancer cells but appears to decrease cellular proliferation, questioning the role of SPC and OGR1 in transformation of ovarian epithelium [Xu et al., 1995]. LPC, LPS, and PAF can activate ovarian cancer cells, but their effects on physiological responses are unclear [Xu et al., 1995]. PA, which is both a precursor and a product of LPA metabolism, can modestly increase proliferation of ovarian cancer cells [Xu et al., 1995]. The complete spectrum of responses to the different lipids, lysolipids, and isoforms in ovarian cancer will require a thorough evaluation of the levels and responses to these moieties. Several may prove both to be functionally important and optimal targets for therapy.

The responses of ovarian cancer cells to LPA have been extensively evaluated (see Fang et al., 2002; Mills et al., 2002 and references therein). At concentrations to which ovarian cancer cells are exposed, LPA can modestly increase the proliferation of ovarian cancer cells. Its pathophysiological role in ovarian cancer may link more tightly to the metastatic cascade than to cell growth as in addition to the effects on the production of neovascularizing factors described above, it increases invasiveness through altering cytosekeletal organization, increasing cellular motility, increasing produc-

tion and action of urokinase plasminogen activator (uPA), and activity of metalloproteinases (MMP2 and MMP9), all critical components in the metastatic cascade. For cells to metastasize, they must detach from their underlying matrix and move to a new site. The vast majority of tumor cells die from anoikis, a form of apoptosis. LPA is highly protective from both apoptosis and anoikis. The ability of LPA to prevent apoptosis may also contribute to the poor outcome in the disease through induced resistance to the main drug used in ovarian cancer cisplatin [Fang et al., 2002; Mills et al., 2002].

The mechanisms by which LPA mediates it functional effects on ovarian cancer cells are only beginning to be delineated. In ovarian cancer cells, LPA through binding to its cognate receptors induces increases in cytosolic free calcium, Rac, Rho, and Rock activation, activation of phospholipase C and D, as well as activation of tyrosine kinases and the PI3K and Ras/MAP signaling cascades [Fang et al., 2002; Mills et al., 2002; Feng et al., 2003]. These in turn lead to activation of NFκB, AP1, and AP2. The roles of specific signaling pathways in specific responses to LPA remain to be delineated.

Almost half of all drugs in current use target members of the GPCR family of receptors making the Edg family of LPA receptors attractive targets for therapeutic development. By analyzing the structure−function relationships of the Edg receptors using yeast, insect and mammalian cells, we and others are beginning to develop receptor selective agonists and antagonists for LPA1, -2, and -3 [Fang et al., 2002; Lynch and Macdonald, 2002; Mills et al., 2002; Feng et al., 2003]. As LPA1 may be a negative growth regulator, agonists of LPA1 may decrease viability and growth of ovarian cancer cells. However, as LPA1 can mediate cellular motility, this could come at the consequence of increased tumor spread. In contrast, LPA2 and -3 can increase motility, production of neovascularizing factors and induce proliferation, respectively, suggesting that antagonists may demonstrate optimal activity. Further, LPA2 is implicated in the production of neovascularizing factors and in particular IL8, which may define the aggressiveness of ovarian cancer [Fang et al., 2003]. LPA3 is particularly appealing as it is selectively activated by the subtype of LPA (with unsaturated fatty acyl chains) that

are found at high levels in ascites from ovarian cancer patients [Bandoh et al., 1999; Xiao et al., 2001; Fang et al., 2002; Mills et al., 2002; Feng et al., 2003]. Indeed, LPA subtypes with unsaturated fatty acyl chains are highly active on ovarian cancer cells. LPA receptors are expressed by multiple cell lineages including fibroblasts, platelets, and brain cells [Fang et al., 2002]. However, the observation that LPA3 is selectively elevated in ovarian cancer cells and that ovarian cancer cells are particularly responsive to specific species of LPA (polyunsaturated fatty acyl chains) suggests that inhibitors based on these forms of LPA may demonstrate specificity for ovarian cancer cells. Taken together, ovarian cancer appears to be driven through the production and action of LPA. Thus inhibitors of LPA production or action may provide an effective approach to the therapy of this disease.

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